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Multiple endpoints of endocrine disruption in gravid watersnakes (Colubridae:Nerodia) as a function of ingestion of a common herbicide

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Multiple endpoints of endocrine disruption in gravid watersnakes (Colubridae: *Nerodia*) as a function of ingestion of a common herbicide

By

LORIN ANNE NEUMAN-LEE

THESIS

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ABSTRACT

Ecotoxicological studies that focus on a single endpoint might not accurately represent the true ecological effects of a contaminant. Exposure to atrazine, a widely-used herbicide, disrupts endocrine function and sexual development in amphibians, but studies involving reptilian species are lacking. This study examines several effects of atrazine ingestion on gravid females and neonates exposed *in utero* of the Northern Watersnake (*Nerodia sipedon*). Twenty-five gravid *N. sipedon* were collected and kept in the lab for the entirety of their gestation period. Each snake received one of four doses of atrazine: control, 2, 20, or 200 ppb. Blood samples were drawn each week to quantify the estradiol levels, and female survival was monitored throughout the study. Following birth, the neonate morphometrics, sex ratio, and percent stillborn were recorded. Data analyses showed that atrazine ingestion potentially disrupts estradiol production in females, inhibits the immune response, alters sex ratio, and causes a higher proportion of stillborn neonates. Findings such as these emphasize the need for additional research involving other reptile species and multiple endpoints in order to determine the full range of ecological impacts that are manifested by contaminant exposure.

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INTRODUCTION

"No witchcraft, no enemy action had silenced the rebirth of new life in this stricken world. The people had done it themselves." Rachel Carson, 1962

The use of anthropogenic chemicals for controlling pest species became widespread in the middle of the 20th century. Soon after this boom, negative effects in non-target species became apparent. The first organisms that were studied in detail included piscivorous birds such as eagles and osprey (Ames 1966; Stickel et al. 1973). Researchers examining the causes of population declines in these birds and found that chemicals such as dichlorodiphenyltrichloro-ethane (DDT) were weakening the integrity of eggshells and causing mortality of the embryos due to shell breakage (Coon et al. 1970; Ratcliffe 1970). Rachel Carson's now-famous book, *Silent Spring*, brought the harmful effects of DDT and other chemicals to light in 1962 and the field of ecotoxicology emerged.

While DDT was banned in the US in 1974, the number of anthropogenic chemicals used has grown worldwide and, with little or no regulation, continues to cause public health concerns (Grier 1982; Pimentel et al. 1992; Landrigan et al. 1999). These chemicals are used for everything from biological control to the manufacture of plastics and paper products. With so much development, the governmental agencies created to oversee the potential impact of these chemicals have become overwhelmed, and acute

versus chronic effects are the focus of laboratory studies that are used to justify the safety of such chemicals (Kavlock et al. 1996).

Many contaminants are now being recognized as endocrine disruptors, defined as compounds that influence the development and/or functioning of an organism through chemical mimicry or interference. Such compounds, often organic, reduce long-term fitness by negatively impacting behavior, survival, and reproductive physiology (Daston et al. 2003; Hayes 2005), but are often difficult to detect using standard toxicological assays. These effects can be detected in an individual, its progeny, or the population as a whole (Bigsby et al. 1999, Colborn and Thayer 2000, Crews et al. 2000)

Endocrine Disruption Chemicals

In the traditional toxicological model, the threshold value for a given chemical is based on the assumption that increasing the dose produces an increased response. This relationship is typically linear and is used to determine LD_{50}, or the concentration at which 50% of the exposed population dies. This model can also be used to determine other endpoints based on a variety of acceptable parameters (*e.g.*, what concentration causes headaches versus that which causes death; Newman and Unger 2003). The U.S. Environmental Protection Agency (USEPA) has used this model to regulate chemicals such as benzene, arsenic and cadmium that are toxic to humans (Travis and Hattemer-Frey 1988).

As the ability to measure smaller concentrations of chemicals developed, a different pattern emerged indicating that accepted limits of the traditional dose-response model might not accurately reflect exposure risk (Kavlock et al. 1996). This new pattern is characterized by an inverted U, or nonmonotonic, shape. There is a high response at

low concentrations with a rapid decrease in response at intermediate concentrations. The response increases again at higher concentrations (Fig. 1; Bigsby et al. 1999; Calabrese 2001; Newman and Unger 2003). Endocrine disruptors negatively impact the organism in a holistic manner, and are generally not well understood. The balance that the endocrine system maintains within an animal's body, using a series of feedback systems, explains this phenomenon. Studies conducted on endocrine disruptors that display this pattern are sometimes disregarded as anomalous data because of the difficulty in fitting the traditional dose-response model (Crews et al. 2000; Du Preez et al. 2008). While the EPA examined the possibility of screening chemicals for this nonmonotonic response curve, the idea was rejected in 2002. Moreover, chemicals regulated by the Toxic Substances Control Act (approximately 70,000) are not subject to low-dose testing (Daston et al. 2003).

Low-dose effects occur through biological processes (Calabrese 2001). The study of this phenomenon is termed "hormeosis," or "the ability to stimulate biological processes by low levels of toxic agents" (Stebbing 2003, pg. 463). Many researchers argue that these agents are not the direct cause of these responses, rather that the elicited response is the organism's physiological attempt to neutralize the unknown substance (Stebbing 2003).

The mechanisms behind endocrine disrupting chemicals are still being elucidated. This task has been difficult given the wide variety of chemicals and possibilities of action on different endocrine organs and systems. Research has shown negative effects in hormone-mediated processes from thyroid hormone control to protein synthesis to pituitary functioning (Cooper et al. 2000; Colborn 2002; McCarthy and Fuiman 2008).

One of the examined mechanisms in the reproductive system is the action of compounds that interfere with normal sex steroid functions and/or rates of synthesis. Many of these compounds act on the estrogen or androgen receptors in the cells of organisms, which cause an imbalance in the amount of sex steroid produced. The effects of these compounds are most pronounced during critical developmental periods in an animal's life when sex steroids control organogenesis and cell differentiation (vom Saal et al. 1997).

Masculinization occurs throughout the developmental period and relies completely on hormonal influences. Because traits associated with maleness are activated by hormones (in humans), genetic males are considered more susceptible to endocrine disruption than genetic females (Sharpe 2006). The disruption of this process can be subtle and not detected until sexual maturity. Although the cause is not known to be natural or anthropogenic, there is an emerging trend of male disorders associated with endocrine disruption in humans (Toppari et al. 1996). While data on humans are difficult to analyze because of confounding variables, studies have shown that decreasing sperm counts due to a decrease in the formation of Sertoli cells, testis cancer, cryptorchidism (failure of testes to descend), and hypospadias (urethra on underside of penis) are becoming more prevalent (Sharpe and Skakkebaek 2003).

Because deformities of the primary sex characteristics can be influenced by multiple factors (genetic, maternal environment and behavior, and environmental) it is difficult to parse out the effects of each factor (Fig. 2; Sanderson 2006; Sharpe 2006). These factors influence the development of the testes, which are the major producers of testosterone. If the testes are malformed, altered testosterone levels will further cause

reproductive deformities. This can cause many problems because the estrogen/androgen balance is extremely delicate and the fluctuations in it can cause reproductive malformations (Rivas et al. 2002). Contaminants might also mimic estrogens, bind to estrogen receptors, alter receptors, and/or change functioning of other hormonal processes (Danzo 1997; Tabb and Blumberg 2006). For example, androgen functioning and androgen receptors may also be suppressed by high levels of estrogens or estrogenic-like compounds (Haavisto et al. 2001; Williams et al. 2001).

There are a number of examples of endocrine disruption in organisms that have been used as models for further research in this field, especially related to an alteration of male sexual characteristics. These examples include the feminization of California Gulls by DDT (Fry and Toone 1981), the demasculization of Florida panthers (Facemire et al. 1995), and the decreasing of sex steroids and male sexual characteristics in white suckers after exposure to pulp mill discharge (Munkittrick et al. 1994).

The physiological process of regulating estradiol and its effects is frequently examined in studies of endocrine disruption. There are numerous hormones that have been examined in only a handful of studies, however, and have critical functions in the development or maintenance of an organism (Propper 2005). Many additional measures of potential endocrine disruption that are not direct measurements of hormones can include measuring stress protein response (Snyder and Mulder 2001), protein synthesis and metabolism (McCarthy and Fuiman 2008), lipid synthesis and metabolism (Heindel 2003), neural functioning (Rodriguez et al. 2005), DNA methylation (Tabb and Blumberg 2006), behavior (Gioiosa et al. 2007), and changes in nuclear (Danzo 1997) and hormonal receptors (Crews et al. 2000).

Endocrine Disruption and Immunotoxicity

Within the framework of the endocrine disrupting model, mortality for an individual can still be assessed. Mortality from contaminants may not only be due to the toxicity at high doses, but can be linked to a suppressed immune response. Very few contaminants were screened specifically for this parameter until 2000, when the USEPA mandated that new products must undergo testing for potential alterations to an organism's immune function (Vos and Moore 1977; Ahmed 2000; Pruett et al. 2003).

Some research has been done on chemicals to evaluate their effect on the immune response and this work has revealed that a wide variety of contaminants, from lead to organochlorine insecticides, negatively affect the immune system (Wassermann et al. 1969; Koller and Kovacic 1974). One example that illustrates this approach is the focus on the spread of the chytrid fungus (*Batrachochytrium dendrobatidis*) in amphibian populations. Recent research on this system has examined the role that contaminants may have in an immunosuppression response that makes amphibians more susceptible to the deadly fungus (Christin et al. 2004; Davidson et al. 2007).

Some toxicants have been shown to lower an animal's immune response in a variety of ways. A major component of immunosuppression is the increase in adrenocortical hormones. Produced in the adrenal glands, these hormones are released in response to stress. While short bursts of these hormones are necessary to mobilize energy reserves to cope with an immediate stressor (*e.g.*, escaping a predator), long-term exposure can have a variety of negative impacts, such as damaging and depleting the cells of the major centers of the immune response—the thymus, spleen, and lymph nodes (Morse et al. 1975). While this measure can aid in understanding the effects at an

individual level, studying the reproductive functions of organisms exposed to contaminants might be more important for understanding the effects at a population level (Barlow et al. 1999).

Atrazine

Since being registered commercially in the United States in 1959, atrazine, an organic triazine compound (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine), has been utilized as a pre-emergent herbicide in corn, sorghum, and sugar cane fields. It is dispersed aqueously to control broadleaf plants. The majority of atrazine is applied to fields planted with corn, one of the predominant crops of the Midwest (Solomon et al. 1996; Solomon et al. 2008). Atrazine's mechanism of action is to inhibit photosynthesis by blocking the electron transport chain of photosystem II. This prevents the conversion of solar energy (photons) to electrical energy within the chloroplast (Fuerst and Norman 1991).

Although atrazine is applied directly to the fields, high levels are found in water bodies, rain and snowpacks, even in areas hundreds of kilometers from application sites (Hatfield et al. 1996; Mast et al. 2007). It is one of the most commonly found anthropogenic chemicals in the atmosphere over Canada with peak concentrations in the Spring and Summer (Brun et al. 2008). The amount of atrazine applied in Canada is less than 1% of that applied in the United States (229 tonnes to 318,000 tonnes) – thus, the amount of atrazine in the atmosphere above the US is likely to be much higher (Brun et al. 2008; USEPA 2009).

Atrazine is persistent in the environment due to its physical properties. Because its structure is an s-triazine ring, it is resistant to microbial degradation, although research

has focused on the potential of cultivating microbial enzymes to degrade atrazine (Komang et al. 2002). Sunlight, aerobic conditions and decreasing pH induce chemical degradation of atrazine's structure both independently and, to a greater extent, synergistically (Solomon et al. 1996). Because of the complicated interactions of these three factors, it is difficult to accurately determine atrazine's half-life in the environment. Studies have shown variation in half-life from 1 day to 244 days, depending upon conditions (Solomon et al. 1996). Some estimates place degradation rates in more neutral and basic rivers, such as those found in the Midwest (Kalkhoff et al. 2000), at several months (Comber 1999). Lotic environments are the likely sites of chronic exposure to non-target organisms because of these properties (Solomon et al. 1996).

Although the mechanism of degradation is similar to that occurring in aqueous media, the degradation rate of atrazine is typically slower in soil environments. Atrazine's degradation rate decreases in deeper soils (≥120 cm) due to atrazine's leach potential (Kruger et al. 1993). Soils with greater structure (such as highly organic soils) decrease the degradation rate while looser soils (less sand content) facilitate the rapid movement of atrazine from one area to another (Graymore et al. 2001).

Atrazine does not readily bioconcentrate or bioaccumulate due to its low K_{ow}, which is calculated by taking the ratio of the concentration of the chemical in water and the concentration of the chemical in octanol (which represents organic matter) at a constant temperature. A low K_{ow} indicates high water solubility and typically a low bioconcentration factor for organisms (Newman and Unger 2003). Atrazine is water soluble (Solomon et al. 1996) but may also be able to accumulate to some extent in tissues with a high lipid concentration (Du Preez and Van Vuren 1992). Uptake of

atrazine to a steady state in zebra fish happens quickly. After 24 h, 30% of the steady state concentration remained in the fish adults and in juveniles, and only 4% of atrazine had been transformed to metabolites after 24 h (Görge and Nagel 1990). Atrazine toxicokinetics performed on amphibian larvae showed that individuals both absorbed and excreted atrazine quickly (Edginton and Rouleau 2005).

The metabolites of atrazine, desethylatrazine (DEA), desisopropylatrazine (DIA), diaminochlorotriazine (DACT), hydroxyatrazine (HA), desethylhydroxyatrazine (DEHA), and desisopropylhydroxyatrazine (DIHA), however, can persist in body tissues for longer periods of time (Solomon et al. 2008). Not only do these products form and degrade at different rates (Winkelmann and Klaine 1991), the mechanisms of action as well as the effects of most of these metabolites are poorly understood.

Atrazine as an Endocrine Disruptor

Little debate remains that exposure to high concentrations of atrazine produces disruption of endocrine function (Cooper et al. 2000; Solomon et al. 2008). The endocrine disrupting properties resulting from low level exposure to atrazine (following the traditional endocrine disrupting model), however, have become the center of debate among the scientific community (Hayes 2004; Solomon et al. 2008). To address this, the USEPA initiated a review in 1994 by The Institute of Wildlife and Environmental Toxicology at Clemson University of atrazine's impact on human health and ecosystems. During this review, the USEPA set the maximum contaminant level for atrazine at 3 ppb. These levels are often found in public drinking water sources (Biradar and Rayburn 1995; Chapman and Stranger 1995). A similar review by the European Union resulted in the

banning of atrazine in 2004 because of the mounting evidence of atrazine's impact on the endocrine system (Sass and Colangelo 2006).

One effect resulting from atrazine exposure, well-studied in several taxa, is the sex ratio in neonate animals exposed during development. Because of their prolonged exposure to aquatic contaminants, fish and amphibians have been used frequently to study the impacts of atrazine on sex ratio and the morphology of the reproductive system. For example, male frogs (*Lithobates* sp.) exposed to low levels of atrazine display testicular dysgenesis, hermaphroditism (including the presence of oocytes within testes), and sex reversal (Hayes et al. 2002a, 2003, 2010). Secondary sex characteristics, such as larynx size in male frogs, are also negatively affected by atrazine exposure at low doses (Hayes et al. 2010). Many studies have shown deviations from the expected 50:50 sex ratio (often becoming female-biased) in amphibians exposed to low levels of atrazine (e.g., Langlois et al. 2009).

Other studies, involving mammals, crocodiles, and turtles, have not shown that sex ratio is affected by exposure to atrazine during development. While several researchers have reported a negligible impact (Gammon et al. 2005; Neuman-Lee and Janzen 2010), at least one (Willingham 2005) showed that turtles with temperature-dependent sex determination produced a female-biased sex ratio most likely caused by an interaction between temperature and atrazine.

The feminizing properties associated with atrazine exposure in some taxa have prompted more recent research examining the hormonal interactions affected by the pesticide. One proposed action impacted by atrazine is the induction of aromatase, an enzyme that naturally converts testosterone and dehydroepiandrosterone (DHEA) to

estradiol (Fig. 3; Matsushita et al. 2006; Sanderson 2006). While atrazine itself has a low affinity for androgen and estrogen receptors, it shows affinity to the steroidogenenic factor I (SF-I) which induces the production of aromatase (Fan et al. 2007a, 2007b). However, not all research has shown aromatase production to be influenced by atrazine (*e.g.*, Hecker et al. 2005).

Another negative impact of atrazine exposure is the reduction of ligand-binding to androgen receptors (Danzo 1997). Estrogen-receptor mRNA in brain cells has also shown to be increased by atrazine exposure (Langlois et al. 2009). Either an increase in aromatase concentration or a reduction in binding to androgen receptors will simultaneously reduce plasma testosterone and increase female sex hormones, such as vitellogenin and estradiol (Spanò et al. 2004). These alterations in hormone concentration are most severe at low doses of atrazine (Spanò et al. 2004; Coady et al. 2005; Hayes et al. 2010).

A suppression of luteinizing hormone (LH) has also been linked to atrazine exposure. LH serves in the negative feedback loop to inhibit estrogen production (Fig. 4) but also participates in a positive feedback loop prior to ovulation. LH spikes are required for ovulation (Hadley and Levine 2003). In several studies, rats dosed with atrazine have suppressed LH. The consequence of this suppression is a reduction in successful implantation of eggs (Cummings et al. 2000). This suppression is not due to the binding on the estrogen receptors (McMullin et al. 2004). Rather, the cause may lie in the hypothalamic center, which has implications for many other hormones due to the extensive control that hormones from the hypothalamus have on the production and secretion of other hormones (Cooper et al. 2000).

Besides the evidence of disruption to the sex steroid pathways, atrazine has been linked to decreased long-term survival. Atrazine may act as an immunotoxicant by increasing corticosterone levels and decreasing thymus cells (Pruett et al. 2003). Few studies have examined atrazine's impact on survival and/or immunotoxicology for longer than one week, or in a range of ecological settings (Solomon et al. 2008). However, studies on amphibians have linked low levels of atrazine exposure with reduced survival (Storrs and Kiesecker 2004; Rohr et al. 2006). This trend is, again, contradictory to the dose-response toxicology model but is supported by the endocrine disruption toxicology model (Stebbing 2003).

Hormone Control in Embryonic Development

Estradiol and testosterone are the primary hormones involved in sex differentiation. The "default setting" for sexual development is female and testosterone secretion is required in order for an organism to develop male sexual characteristics. Although an animal's gender might be determined by different mechanisms (genetic sex-determination and temperature sex-determination), testosterone is required to control the development of male physical characteristics and behavior (Hadley and Levine 2003).

Testosterone and DHEA are produced in the adrenal cortex, the Leydig cells of the testes, the prostate gland, thecal cells of the ovaries, and the placenta. Its production relies on enzymes in the adrenal cortex that transform cholesterol into pregnenolone and progesterone to 17α-hydroxypregnenolone in the mineralcorticoid pathway, and to 17α-hydoxyprogesterone in the glucocorticoid pathway. Once entering the androgen pathway, these two steroids are converted to DHEA and androstenedione which are converted to testosterone. Any inaccuracy in these steps can interfere with the production

of testosterone (Hadley and Levine 2003). Testosterone's primary function in development is to form the Wolffian Ducts, which will develop into the epididymis, seminal vesicles, and vas deferens, and to be converted by 5-α-reductase into dihydrotestosterone, which regulates the development of the penis, scrotum, and prostate glands (Hadley and Levine 2003).

Because it is a steroid produced by the conversion of testosterone and/or DHEA, estradiol has been the focus of many studies. Estradiol production is regulated by a negative feedback loop involving the hypothalamus, anterior pituitary gland, adrenal cortex, and target organs (*e.g.*, ovaries; Fig. 4). With rising levels of estradiol, the hypothalamus' production of gonadotropin-releasing hormones (GnRH) is suppressed. The suppression of the GnRH, as well as the direct suppression of increasing levels of estradiol, causes a decrease in the anterior pituitary's production of LH and follicle-stimulating hormone (Bronson 1981).

Sex Hormones and Ecotoxicology in Reptiles

Research concerning the sex hormones of reptiles has focused on a relatively small number of species (Moore and Lindzey 1992; Whittier and Tokarz 1992) which makes applying any generalizations of endocrine disruption to reptiles difficult. Part of this challenge stems from the pattern of dissociated reproduction that is often practiced by reptiles – this occurs when the gonadal activity peaks at a different time than sexual activity (*e.g.*, Whittier et al. 1987). Male hormonal fluctuations in *N. sipedon* appear to follow a typical associated reproductive strategy, with testosterone peaking in the early spring during reproduction and in the fall during spermatogenesis (Weil and Aldridge 1981).

Although reptiles are under-represented in ecotoxicological literature, they are ideal model organisms (Hopkins 2000) because they: 1. exhibit high site fidelity; 2. are long-lived; 3. have well-defined behaviors; 4. are relatively easy to maintain in captivity; and, 5. are primarily carnivorous. The most commonly used reptiles for research involving organic contaminants are the Common Snapping Turtle (*Chelydra serpentina*) and the American Alligator (*Alligator mississippiensis*; Guillette et al. 1994, De Solla and Fernie 2004). Ecotoxicological research involving squamates (snakes and lizards) has remained particularly sparse, although the Banded Watersnake (*Nerodia fasciata*) has been the subject of several studies involving exposure to heavy metals (Ohlendorf et al. 1988; Hopkins et al. 1999; Murray et al. 2010).

One well-known study involving reptiles and endocrine disruptors is research conducted at Lake Apopka in Florida. This location is home to many American Alligators (*Alligator mississippiensis*) and was also the site of a major DDT spill in 1980. This research revealed a drastic decrease in penis size between the exposed male alligators and alligators from a control site. Additionally, lower androgen levels were found in the contaminated alligators, which is a potential explanation for the decreased phallus size (Guillette et al. 1996). Not only did the testosterone levels decrease, but the estrogen levels increased up to two times of the levels observed in alligators compared to the control site (Guillette et al. 1994). This work has highlighted extreme effects of endocrine disruption in reptiles.

The Snapping Turtle (*Chelydra serpentina*) has also been a model organism for examining endocrine disruption. In one study, sexual dimorphism was affected by endocrine disruptors (organochlorines), but the sex hormones seemed to be unaffected

(De Solla et al. 1998). However, atrazine may have an impact on male development (De Solla et al. 2006). Snapping turtle eggs have been used to quantify the amount of contamination that occurs within the egg. This technique has provided invaluable information that has increased our understanding of the impact of contaminants on oviparous development (De Solla et al. 2001; De Solla and Fernie 2004).

Natural history of the Northern Watersnake

The Northern Watersnake (*Nerodia sipedon*) is an ideal model organism for ecotoxicological studies given its life history (Hopkins 2000). Additionally, they are found throughout much of the Midwest in abundant numbers (Conant and Collins 1991). Population studies have shown that individuals of this species remain in the same area all year (King 1987), and repeatedly utilize particular microhabitats (Olds 2007).

Nerodia sipedon is a viviparous reptile that typically reproduces every year. However, up to a third of mature females will abstain from mating each year (Weatherhead et al. 1995). The species exhibits sexual size dimorphism with female snakes being larger than similarly-aged males after their second year (Brown and Weatherhead 1999). Sexual maturity is attained at three years for males and three to four years for females (Weatherhead et al. 1995; Brown and Weatherhead 1999). Clutch size has been reported to be between 4 and 99, and larger females tend to produce female-biased neonates in both captive and wild environments, although the overall sex ratio of a population is maintained at approximately 1:1 (Weatherhead et al. 1995).

Nerodia sipedon has been the subject of many behavioral and ecological studies that include topics such as site fidelity (Fraker 1970), reproductive behavior (Mushinsky 1979; Weatherhead et al. 1995), thermoregulation (Brown and Weatherhead 2000),

responses to chemical cues (Scudder et al. 1980), and even the effects of pedestrians on basking behavior (Burger 2001).

Nerodia sipedon is carnivorous, with the majority of its diet being fish and amphibians (King 1993). Although some species of *Nerodia* have been maintained in captivity for periods of several months, researchers have not attempted to keep large numbers of *N. sipedon* in captivity for a period over three weeks (R.B. King, pers. comm.; N.B. Ford, pers. comm.).

While little research has been conducted on organic contaminants and *Nerodia* to date, several studies have analyzed multiple endpoints of contaminant exposure. For example, Murray et al. (2010) estimated heavy metal exposure on *N. fasciata* and correlated exposure with DNA-strand breakage. Other endpoints that have been examined in the context of heavy metal exposure and *N. fasciata* include: standard metabolic rate, bioaccumulation after chronic exposure, liver histopatholoy, and trophic transfer (Hopkins et al. 1999, 2002, 2005; Ganser et al. 2003). Other research on *Nerodia* has focused on using non-destructive sampling in the field to create a representative view of heavy metal load within snake tissues (Hopkins et al. 2001).

Research Objectives

The purpose of this study was to detect endocrine disruption by ingested atrazine in wild-caught *N. sipedon* by measuring plasma estradiol levels throughout the three month gestation period. Atrazine's potential as an immunotoxicant was explored in relation to the survival of the females. To determine effects of atrazine ingestion on the neonates that were exposed *in utero*, comparisons of morphometrics, sex ratio, and

condition (live vs. stillborn) of the neonates were made between the four treatment groups.

MATERIALS AND METHODS

Study Sites and Specimen Collection

During or shortly after their emergence from hibernacula in April 2009, nine female snakes were collected at Lake Charleston near Charleston, Coles Co., Illinois (-88.1468785 long., 39.467139 lat.; Fig. 5). The population density of *N. sipedon* at this location is relatively high, and all life history stages have been observed at this site (Olds 2007). Another 16 female snakes were collected at Lake Mattoon, Cumberland Co., Illinois (-88.48172 long., 39.33337 lat.; Fig. 5). Additionally, seven males from Lake Charleston and five males from Lake Mattoon were captured and brought to the laboratory. Patterns of land use (*e.g.,* row-crop agriculture) within 500 m of either lake and along the watersheds that flow into the lakes make snakes' exposure to atrazine highly probable.

Mass (±0.1 g) and snout-vent length (SVL; ±1 mm) was recorded for each individual using standard methods (Fitch 1987). Each individual was marked uniquely using a medical cauterizer (Winne et al. 2006) and gender was verified using a cloacal probe. Blood was obtained (1 ml) from the caudal vein of each snake for hormonal analysis (described below).

Every snake was housed in a 30x30x60 cm plastic cage, each having newspaper substrate, a water dish, a rock to aid with shedding, and a plastic shelter. Males were immediately paired with females. During this time, water was available *ad libitum*, but animals were not fed. After one week of being paired with a single female, the male was rotated to a new female for an additional week to increase the likelihood of insemination (Riches 1976). After the second week, to further facilitate individual identification, the

males had a passive integrative transponder (PIT tag; 12x2.1 mm) inserted into their body cavity (Fitch 1987; Jemison et al. 1995). Males were then released at their respective sites of capture.

After the watersnakes gave birth, their health was monitored. If there were no major negative signs from the ingestion of atrazine, the female subjects were released 1-2 weeks after giving birth. Before release, all snakes were re-measured and individuals from Lake Charleston were implanted with a PIT tag.

Atrazine Ingestion

Beginning on 1 June 2009, female watersnakes in captivity began to receive one of the following atrazine treatments: 0 (control), 2, 20, or 200 ppb of atrazine. The treatment group to which each female was assigned was randomly selected. Live minnows (*Notropis* sp.; Pana Bait Company, Pana, Illinois) were obtained weekly and injected with 1 ml of atrazine solution (the concentration of which was confirmed using ELISA; see below). The fish were immediately offered to the respective snake for a period of not more than 1 hr to avoid any degradation of atrazine. If the fish were not consumed in that time frame, the female snakes were force fed by palpation to ensure that the embryos within each female were exposed to the appropriate atrazine concentration throughout gestation. Females were fed in this manner twice weekly, such that each individual was fed 10% of her body mass every week. Every week, 1 ml of blood was obtained from the caudal vein for hormonal analysis. The sample was placed on ice for a maximum of 1 hr, until processed further (described below).

In mid- to late August, females were observed closely for signs of parturition. As soon as the neonate snakes were born, they were removed from the mother's enclosure.

The head and neck of each neonate was photographed to allow for unique identification. Within 24 hr of birth, a 0.1 ml blood sample from each live neonate subject was obtained, its mass and SVL were recorded, and its gender was determined using a cloacal probe. Each neonate was housed with its siblings in conditions similar to those previously described for the adults. After 3-4 weeks, the neonates were euthanized by overdosing with inhaled isoflurane, and then placed in a freezer (-20 °C) until further analysis.

The neonates that were stillborn were measured immediately (mass and SVL), probed for sex determination, and then marked dorsally with a unique sequence of white dots. These individuals were frozen (-20 °C) before further analysis (see Appendix II). All neonates, embryos, and undeveloped yolks were weighed and used to calculate relative clutch mass (RCM; Seigel and Fitch 1984; Gregory et al. 1992).

Atrazine Preparation

The atrazine (98.9%, ChemService; West Chester, PA) was dissolved in 1 ml 95% ethanol, which was then mixed with 1 L diH_2O. Using this as a stock solution, the three treatment groups (2, 20, and 200 ppb) were mixed via serial dilution. The control solution was 1 ml 95% ethanol and 1 L diH_2O. Verification samples were confirmed using an enzyme-linked immunosorbent assay (ELISA; see below for description). The concentrations used for this study are all realistic ecological amounts given the proximity of water bodies to agricultural fields (Graymore et al. 2001; Gross 2009).

Blood Sample Preparation

The blood sample obtained from each female subject remained on ice for not more than 15 min before being spun in a microcentrifuge for 10 min at 8,000 rpm, which separated the plasma from the red blood cells. The plasma was extracted and placed in a

new 1.5 ml vial. Within 45 minutes after been separated via centrifugation, the blood and plasma samples were frozen at -20 °C until further analysis. Because there was an inadequate amount of neonate plasma to run each sample individually, the samples were pooled by clutch and by sex (*e.g.*, male siblings' plasma was mixed). Depending on the clutch, plasma from three or four neonates comprised one sample.

Solid Phase Extraction of Estradiol

The plasma was thawed on ice for approximately 20 min and then prepared for the ELISA evaluation by performing a solid phase extraction (SPE; protocol described in Appendix I and modified from Newman et al. 2008). SPE isolates the nonpolar steroid hormones (specifically 17β-estradiol) from water by bonding them to a nonpolar sorbent (in this case, a C_{18}-bonded silica). This process generates Van der Waals forces, which allow for all polar components to be separated from the nonpolar components. When using plasma, this process also allows for the removal of lipids such as fatty acids and triglycerides (Sigma-Aldrich Co. 1998; Newman et al. 2008). A known concentration of 17β-estradiol (Steraloids Inc., Newport, RI) was extracted to determine the percent recovery. The procedure yielded a 72% recovery rate, which is comparable to other studies that have used this method (*e.g.,* Newman et al. 2008).

ELISA for Estradiol

Dilutions were necessary to obtain concentrations between the detection limit of the ELISA kit (2.5 – 25 pg/L) and were performed according to protocol (Abraxis 2008). These diluted samples were shaken for one hour and then analyzed using the 17β-estradiol (E2) ELISA Kit (magnetic particle) manufactured by Abraxis LLC (Warminster, PA). This is a competitive ELISA that binds exclusively to E2 with

uniform monoclonal antibody quality. A conjugate, 17β-Estradiol-enzyme, is used to determine the concentration of E2 in the sample. The lighter the color (lower absorbance) indicates a greater concentration due to the E2 preferentially binding to the sites. The concentrations are determined all at 450 nm, based on a standard curve (Abraxis 2008).

Female Mortality

Throughout the study, each snake was monitored for any signs of weakness, sickness, or other signs that might indicate a decline in health. Approximately three weeks following the first dose of atrazine, some of the subjects were showing signs of infection on the scales and skin. Such individuals were treated by soaking individually in a 10% povidone-iodine (Betadine™) solution, allowing them to bask for approximately 1 hour in a separate tank with a 60 W bulb, and applying a topical antibacterial gel containing neomycin sulfate, polymyxin B, and bacitracin zinc (Neosporin™) to the infected site (Hoppmann and Barron 2007). The progression of this infection was noted until the termination of the experiment or the death of the snake.

Other causes of mortality were noted. Necropsies were performed on any individual that died to determine possible cause of death. If a snake died for a reason unrelated to a scale infection (*i.e.*, the individual had no or minor symptoms of infection, and another cause was apparent), its cause of death was categorized differently from those individuals presenting symptoms characteristic of the scale/skin infection.

Stillborn Neonates

Many female snakes gave birth to fully-developed but dead neonates. The proportion of live to dead neonates for each snake was recorded. Neonates from any

female that died before birthing were not included in the analysis of still-born neonates. In one instance, neonates were removed from a dead female before they died, but they were not included when analyzing the proportion of stillborn neonates.

Statistical Analyses

After testing all assumptions for using a parametric statistics, an analysis of variance (ANOVA) was used to compare the initial masses at capture of all of the females as a function of treatment. An ANOVA was also used to determine if treatment affected the change between initial mass at capture and mass at the end of the treatment (at 3 mo).

The concentration of estradiol was log-transformed to meet the assumption of normality prior to any analyses (PROC UNIVARIATE, SAS v9.0; SAS Institute, Cary, NC). All of the analyses were performed on both the gravid females only (control $n = 5$, 2 ppb $n = 7$, 20 ppb $n = 6$, 200 ppb $n = 5$) and all female snakes (control $n = 7$, 2 ppb $n = 7$, 20 ppb $n = 6$, 200 ppb $n = 6$) combined. For all analyses, values for the gravid females and all female snakes did not differ; therefore, all of the female snakes were used in the analyses.

The two time scales of birth week (back-calculated from when the female gave birth) and treatment week were analyzed using a linear regression (PROC REG). Because of multiple data points for each week, the deviation of linearity was also calculated by using the sum of squares of the linear regression. An Akaike information criterion (AIC; Akaike 1974) calculated the best fit regression model. The best non-linear structure was determined by examining the AIC, ΔAIC, and evidence ratio for up to seven parameters. The ΔAIC is calculated by subtracting the smallest AIC value from

the parameter's value. To determine the evidence ratio, the negative square-root of the ΔAIC is calculated (w_i value). The w_i value for each parameter is divided by the parameter that is next best according to the ΔAIC value to calculate the evidence ratio. A general linear model (PROC GLM) was run with estradiol concentration as the dependent variable and treatment as the independent variable. A mixed model (PROC MIXED) analysis was performed to minimize the effects of repeated measurements of the same individuals within the data.

The mean estradiol concentration was calculated for each female. A t-test was conducted comparing the control to the atrazine-treated females. An additional t-test was conducted comparing the pooled mean of control and 2 ppb treatments to the pooled mean of 20 and 200 ppb treatments because the former treatment levels are below the EPA human consumption limit.

Female mortality and cause of death were analyzed using a logistic regression with treatment and female initial mass as independent variables. Relative clutch mass was calculated by dividing the total clutch mass (both neonates and embryos) by the female's post-birth mass. This was performed only with data from those neonates that were fully developed (as determined by presence of complete scale formation). Analyses of covariance (ANCOVA) were used to compare neonate SVL and mass as a function of treatment group and mass of female at capture. The effect of treatment on the sex ratio of the neonates from each female was determined using a logistic regression.

Differences in the percent of live neonates born to each female snake as a function of treatment were compared with an analysis of variance (ANOVA) with a *post-hoc*

Dunnett's test comparing the mean of the control group to that for each of the treatment groups.

RESULTS

Female Morphometrics

As SVL increased in female snakes, so did their body mass ($F_{7,5} = 8.90$, $p = 0.01$). However, the initial mass ($F_{25,22} = 0.69$, $p = 0.42$) and SVL ($F_{25,22} = 0.48$, $p = 0.70$) of the female subjects did not vary between treatments (Table 1).

Estradiol Levels

The SPE procedure yielded a 72% recovery rate. There was no linear pattern between birth week and the concentration of estradiol ($F_{1,198} = 0.88$, $p = 0.35$). Additionally, there was no deviation from the linearity for birth week ($F_{14,198} = 1.12$, $p = 0.35$). Because of this, no further analyses were run on birth week. The relationship between treatment week and estradiol levels was nonlinear ($F_{1,234} = 0.12$, $p = 0.73$), with a significant deviation from linearity ($F_{11,234} = 5.25$, $p < 0.0001$). When fitting the non-linear models, the AIC and evidence ratio showed that the quartic (5 parameters) model was the best fit, although the quartic, quintic, and sextic (5-7 parameters) models fit the model best as a group according to the AIC, ΔAIC and evidence ratio measures (Table 2). This non-linear pattern can clearly be seen in the fluctuation of the estradiol concentration from week to week (Fig. 6).

The GLM analysis showed no relationship between treatment and the concentration of estradiol ($F_{3,343} = 1.31$, $p = 0.27$). The concentration of estradiol was also not affected by whether or not the female was gravid ($F_{1,343} = 0.48$, $p = 0.49$). The best-fit mixed model was the base model (variance component). Two covariance structures (compound symmetry and auto-regression) were similar to the variance component statistics. The mixed model output was the same as the GLM analysis

indicating that the inter- and intra-individual variances were similar. The t-test comparing the means of the control snakes' estradiol concentrations to the snakes' plasma concentration in the other three treatment groups showed that there were no differences between the means ($t = 0.93$, $p = 2.07$; Fig. 7).

Female Mortality

The subject mortality over the duration of the study was not affected by the concentration of atrazine received in the fish diet ($\chi^2 = 6.70$; $p = 0.46$). Among all treatment groups, four different causes of death were determined (Table 3). The primary cause of death for the snakes was an infection that spread rapidly on an individual's scales (Fig. 8). The initial symptoms included small to medium (0.5 – 1 cm) pustules forming between scales on the ventral side followed by deterioration of the scales, typically starting around the regions of the neck and cloaca.

If the infection advanced (not in all cases), symptoms became more systemic, influencing the musculature. These symptoms included: 1) complete loss of scales and skin, resulting in exposure of flesh, especially around the neck; 2) weakness of muscles (as determined by palpation and body movements; and, 3) poor righting response (requiring more than 30 sec) followed by death. Necropsies performed on individuals that died in the advanced stages of this infection revealed no obvious internal malfunction. Larger snakes that were treated made up a higher proportion of the snakes that died from the infection ($\chi^2 = 32.22$, $p = 0.003$, $R^2 = 0.63$; Fig. 9).

Reproductive Output and Morphometrics

There were no differences in RCM as a function of treatment ($F_{17,14} = 0.44$ $p = 0.73$; Table 4). The sex ratio of the neonates born to these females did vary as a function

of treatment, however, with more male offspring being produced by females exposed to the 200 ppb atrazine treatment ($\chi^2 = 8.23$, $p = 0.04$; Fig. 10). The mass of the neonates did not differ between treatment ($F_{3,9} = 0.97$, $p = 0.54$) or as a function of the mother's initial mass ($F_{1,9} = 1.00$, $p = 0.42$). The SVL also did not differ as a function of treatment ($F_{3,9} = 2.55$, $p = 0.29$) or mother's mass ($F_{1,9} = 1.63$, $p = 0.31$; Table 5).

Stillborn Neonates

The concentration of atrazine in the diet that gravid subjects received during gestation appeared to influence the proportion of neonate snakes that were stillborn ($F_{12,9} = 3.13$, $p = 0.08$; Fig. 11). Specifically, female snakes in the 20 ppb treatment were less likely to produce live offspring than those in the control group. The interaction between the sex ratio and treatment also had an effect on the proportion of neonates that were stillborn, with males being stillborn more frequently than females ($F_{12,8} = 5.31$, $p = 0.02$).

DISCUSSION

The balance between hormones and development is an important area of ecotoxicology because of the potential long-term effects that result from exposure to endocrine-disrupting chemicals. Fluctuations from the normal levels of hormones can impact both organizational and activational development that alters physiology and behavior. Furthermore, using model organisms such as watersnakes, provides unique insight into the effects of atrazine exposure on a common organism that is found in heavily-exposed areas. This study tests a variety of endpoints using an ecologically-relevant means of delivering atrazine – a manner similar to that which snakes experience when ingesting their primary prey item in the field.

Plasma Estradiol

While many studies have used radioimmunoassay as a method of comparing and/or quantifying estradiol levels in reptiles (Whittier et al. 1987; Taylor et al. 2004), the results of this study show that an SPE extraction, followed by an ELISA, can be an effective method to compare estradiol levels in snake plasma. The lack of variation between individuals in each treatment demonstrates that, although the ELISA was not verified by gas chromatography, the relative level of estradiol detected was consistent based on the fact that the similar patterns of estradiol fluctuation were observed for each snake and each treatment (Fig. 6 and Fig. 12).

One of the interesting results was that the non-gravid females had similar levels of estradiol in their plasma when compared to the gravid females. This finding is contradictory to many studies on snake reproduction (*e.g.*, Whittier et al. 1987, Taylor et al. 2004), and might be caused by natural stochasticity because only three individuals

were not gravid and therefore the sample size was small. Additionally, evidence of either retention or expulsion of unviable or dead embryos has been provided for squamates (Blackburn et al. 2003; Blackburn 2006). Because of this, some females in this study may have been gravid for part of the gestation period and aborted the embryos. Because the only confirmation of gravidity was palpation and whether the female gave birth, a snake may have been gravid without being detected.

The non-linear relationship between treatment week and estradiol levels indicated that atrazine exposure explained some of the variance between treatment groups. The non-linear nature of this interaction is consistent with the endocrine disruption model (Fig. 1). Interestingly, the concentration of estradiol did not model similarly when compared to the gestation week. A greater sample size could have aided in determining a more quantifiable pattern for both birth week and treatment week because each snake's estradiol levels had a high amount of variation from week to week.

An important problem in interpreting these data is the documented inter-individual variability in hormone levels. Although no study has examined the variability in estradiol levels in female *N. sipedon*, the androgen levels in male *N. sipedon* can be inconsistent across individuals, although each individual follows a similar temporal pattern (Weil and Aldridge 1981). In *T. sirtalis*, extensive surveys of females have shown a great deal of individual variation in estradiol and progesterone levels (Whittier and Tokarz 1992).

In the present study, the complex relationship between treatment week and estradiol level might be caused by the differential depression of estradiol from corticosterone produced as a result of atrazine exposure. Corticosterone is a

glucocorticoid hormone that is released in response to stressors, such as being maintained in captivity and handling (Mathies et al. 2001). Research has shown that exposure to contamination induces an increased release of corticosterone in alligators (Gunderson et al. 2003). While prolonged exposure to elevated levels of corticosterone can have pernicious effects on an organism, it can also have the effect of depressing estradiol production in reptiles (Elsey et al. 1991; Moore et al. 2000). Because corticosterone is synthesized using the same basic compound as estradiol (cholesterol), this might indicate a limited amount of starting material that is directly available (Hadley and Levine 2003). A negative relationship between corticosterone and estradiol might have necessarily decreased the concentration of estradiol, making a clear connection between exposure and estradiol levels difficult to detect.

Whittier (1987) determined that stress in captivity did not alter the levels of estradiol in female *T. sirtalis parietalis*. This conclusion was formed from analyzing the estradiol levels in both wild and captive populations. It should be noted, however, that the captive animals in Whitter's study were overwintered in the lab and might have become habituated to captive conditions.

It is important to note that elevated levels of a specific sex hormone are not always correlated with sexual development during exposure to contamination. In fact, there can be measurable differences in sexually dimorphic traits between individuals exposed to a contaminant and individuals that are not exposed, even when there is no difference in testosterone and/or estradiol. In the Snapping Turtle (*Chelydra serpentina*), precloacal length, which is greater in males than females, was reduced in males after developing in a contaminated area. However, the testosterone and estrogen plasma levels

were not altered. It is unclear as to the precise control mechanism that caused this relationship, but it may be related to hormone receptors or hormones that were not measured (De Solla et al. 1998). This also may be explained by the phenomenon of temperature-dependent sex determination that snapping turtles exhibit. While estrogen induces the female sex, there is a distinct interaction with temperature that may confound this relationship (Bergeron et al. 1994).

While there are many hypotheses about the action of atrazine on estradiol, there have been no studies on atrazine exposure in reptiles and hormones. The strongest body of available research has been conducted on amphibians and remains largely inconclusive. Some of this research has shown that estradiol levels are lowered in males exposed to low levels of atrazine (*e.g*., Coady et al. 2005). Hayes et al. (2006) demonstrated that atrazine exposure produced very similar effects to exposure to exogenous estradiol, indicating that atrazine may elevate levels of estradiol. Stoker et al. (2000) provided evidence that estradiol increases after exposure to atrazine at certain concentrations. There have not been any studies that have shown the relationship over a longer period of time (> 1 week) between atrazine and estradiol levels.

The results of this study indicate that, in *N. sipedon*, there might not be an induction of aromatase as a function of atrazine exposure. Rather, there might be an effect on steroid synthesis and/or steroid conversion that were not detected by solely measuring estradiol in the blood. For example, many enzymes that aid in the steroidogenic process, such as a variety of cytochrome P450 enzymes, have been shown to be affected by endocrine disruptors but the effect of atrazine on them has not yet been

examined (Sanderson 2006). Additionally, the interaction between all of the intermediate steroid products has not been examined.

Female Survival

Another endpoint examined in this study was the survival of the females over the three-month period. This is a more traditional endpoint for toxicological studies but, in spite of the fact that mortality was not affected by atrazine treatment, the results indicated a pattern that follows the endocrine disruption model. The majority of the deaths were from the infection, but two individuals in the control group died from events that were potentially stochastic in nature (Table 3). A larger sample size, especially within the control group, would have helped determine if the atrazine affected mortality as a whole. Although nearly every animal from every treatment was affected by the initial stages of the infection, subjects in the lowest treatment of atrazine (2 ppb) experienced a higher rate of mortality from the infection than those in the control group. This indicates that atrazine might have had an impact on the immune system of the snakes.

Atrazine ingestion, coupled with the stress of captivity, might have elevated levels of corticosteroids. While corticosterone might be responsible for these effects, the tropic hormone, adrenocorticotropic hormone can also have direct effects on the fitness of an organism, such as the destruction of lymphoid tissue (Vos and Moore 1977). The higher death rate associated with the infection could potentially be linked to elevated corticosterone levels. Given that the duration of this study was approximately three months, corticosterone levels in the female snakes could have been elevated for the entire period. Corticosterone levels in female snakes increase before they give birth, (Taylor et

al. 2004). This further supports the hypothesis of elevated corticosterone because the majority of the females died around the time that they gave birth.

All of the individuals in this study were subjected to the same captive environment and approximate handling time. Given that the subjects receiving the highest atrazine concentration had the highest proportion of deaths from infection (Fig. 10), atrazine might have caused a greater increase in corticosterone, which was likely already high due to the subjects being maintained in captivity (Marra et al. 1995; Mathies et al. 2001). Elevated corticosterone levels can sufficiently lower the ability of the immune system to respond to infections, and this might have been the reason that the subjects with the added stress of atrazine ingestion succumbed to the disease (Vos and Moore 1977). This measure highlights a potential disruption in immune functioning that may also be related to the estradiol levels in the females.

Reproductive Output

Control of development and birthing is mediated in large part by estradiol. While the estradiol level measurements might have been affected by the ingestion of atrazine, a more biologically-relevant endpoint is the reproductive output of the individual. One of the proposed consequences of atrazine exposure at low levels has been a skew in sex ratio, with organisms producing more females than males. This outcome is thought to be due to an increase in estradiol levels. However, the bulk of this work has been conducted on amphibians (Hayes et al. 2006; Rohr and McCoy 2010).

Within this study, there were differences between the sex ratios of the treatment groups. Interestingly, the sex ratio was male biased. This finding is supported by Matsushita et al. (2006) who found that atrazine inhibited the regression of the right

gonad in chicks. This regression is necessary for the development of testes. Therefore, female chicks had testis-like structures (non-regressed right gonads) in addition to fully-functioning ovaries. The differences in sex ratio, as well as the disruption of the estradiol levels when analyzed by week of treatment, indicate that sexual development of the neonates might have been altered by the atrazine treatments.

Because there were no differences in estradiol levels as a function of treatment, it is possible that another hormone or mechanism was responsible for the skewed sex ratio in neonate watersnakes. For example, vitellogenin has recently been proposed as a measurement for endocrine disruption because it is induced by estrogen to produce follicles in a mature female (Sumpter and Jobling 1995). While research has focused on estrogenic contaminants and the presence of vitellogenin in males (Cheek et al. 2001), it could have been measured in this study as an indirect measurement of increased or decreased estrogen activity.

While probing the cloaca is a validated measurement for determining the sex of a snake (Fitch 1960), this method might be inadequate to measure small potential differences in size and development of sex organs. Studies that have revealed abnormalities in the sex organs have relied on morphological characteristics as well as histological ones (Hayes et al. 2002b, 2006; Langlois et al. 2009). Most frequently, these histological analyses have been used to show the alterations in the sex organs.

Another indication of abnormal sex organ development is in quantifying male testicular oocytes, or testicular ovarian follicles (TOFs). The appearance of such structures does not normally occur in genetic males. Several studies have demonstrated an endocrine disruption model pattern for the development of TOFs. For example, the

number of TOFs was elevated after exposure to 10 μg/L atrazine and was lower at 1 and 25 μg/L (Du Preez et al. 2008). Not all studies have demonstrated this interaction, but it is still an endpoint that is used for atrazine and testes development (Jooste et al. 2005). In this study, TOF counts were not made, but could have been useful for analyzing reproductive development.

Finally, because studies on *N. sipedon* populations (both captive and free-living) have revealed a non-biased sex ratio (50:50 ratio M:F; Weatherhead et al. 1998), the male-biased sex ratio observed in this study is unlikely to be a natural phenomenon. Larger female *N. sipedon* tend to produce more females, but the statistical analysis in this study controlled for this feature of their natural history. This phenomenon is currently unexplained, but this bias towards female offspring might be caused by larger watersnakes having the ability to allocate more resources to the neonates and producing females (Weatherhead et al. 1998). However, the sex of the neonate is typically unaffected by its size at birth (Weatherhead et al. 1998; Gibbons and Dorcas 2004).

Neonate Morphometrics

The size of newly-hatched turtles is not affected by exposure to atrazine during development (Neuman-Lee and Janzen 2010), which is consistent with the findings of this study. The only effect on neonate watersnake mass and SVL was the mother's size, which is contradictory to other studies on *N. sipedon* (Weatherhead et al. 1998; Brown and Weatherhead 1999). The mother's size may have affected the neonates' morphometrics due to the differences in location of the population used by Weatherhead et al. (1998, 1999) and this study, and/or due to an unquantified feature associated with atrazine treatment.

Neonate Survival

Ingested atrazine negatively affected the percentage of live births (especially at the 20 ppb treatment level; Fig. 11), a finding that might be a function of a synergistic response to increased corticosterone levels from the atrazine treatment, pregnancy, and the captive conditions. The elevation of plasma concentrations of corticosterone during reproductive periods in both males and females has been shown in many reptiles (Moore and Jessop 2003). Low levels are generally beneficial for reproduction. As the corticosterone levels increase, however, reproduction is inhibited and is often correlated with a decrease in sex steroids (Moore and Jessop 2003; Moore et al. 2005). Robert et al. (2009) documented an increase in still-born neonates as a result of elevated corticosterone plasma levels in *Thamnophis* (a sister Genus to *Nerodia*). However, the toxicity of ingested atrazine on developing squamates has not been examined. Therefore, it is not possible to discount that atrazine had a toxic effect on the neonates.

The proportion of live births differed among clutches having different sex ratios. Female subjects that birthed male-biased litters had lower success of live-birth than those producing female-biased litters, a finding supported by another study on *N. sipedon* (Weatherhead et al. 1998). The negative effect that atrazine had on the proportion of live-birth has implications for the overall population of *N. sipedon*. While Northern Watersnakes are common throughout most of their range, there are other natricine species that utilize similar habitat and prey types and are listed in the United States as federally endangered or threatened due to declining populations (*e.g.*, Copperbelly Watersnake [*N. erythrogaster neglecta*], Lake Erie Watersnake [*N. sipedon insularum*], Giant Gartersnake [*Thamnophis gigas*], and San Francisco Gartersnake [*T. sirtalis tetrataenia*];

US Fish and Wildlife Service 2010). In species with low population numbers, a decrease in live-births can be detrimental to the survival of the population due to the possibility of bottlenecks and/or inbreeding depressions (Újvári et al. 2002).

The sample size for determining the effects of atrazine on the percentage of still-born neonates was greatly decreased by the high mortality of females. It cannot be predicted how many live births and stillbirths would have been produced by these females had they survived.

Use of Multiple Endpoints

While most ecotoxicological studies focus on mortality, this endpoint is unsatisfactory when attempting to understand the complexity of the ecological effects of a contaminant on an organism. In order to truly assess the impacts of a contaminant, multiple endpoints must be analyzed. Using an endpoint that controls reproduction, such as estradiol levels, a framework can be established to examine a host of other endpoints related to reproductive output. Within this study, any single endpoint was inadequate to fully understand of the effects of atrazine ingestion by gravid *N. sipedon*. When all of the endpoints are evaluated (Table 6), however, the negative impacts of atrazine ingestion on reproductive output were elucidated.

Future directions

Little work has been conducted on the effects of exposure during development on future reproductive fitness. Because of logistic complications of maintaining vertebrates in the lab for long periods of time, these analyses are performed infrequently. Some research with amphibians has indicated that there might be an effect on reproductive fitness that continues at least until the F2 generation when individuals are exposed during

development to low levels of atrazine. For example, Du Preez et al. (2008) found that survival of frogs in the F2 generation decreased when exposed to 1 μg/L atrazine. However more research is needed to clarify this relationship.

Exposure to atrazine at different life stages could affect different endpoints. Being wild-caught, the snakes in this study were likely exposed throughout their life span to atrazine as well as numerous other contaminants. Exposure to atrazine at different life stages has shown to cause different effects on individuals in anurans. Allran and Karasov (2001) showed that atrazine exposure had no effect on hatching success from eggs and increased deformities in larvae in three species of anurans (*Lithobates pipiens*, *L. sylvatica*, and *Anaxyrus americanus*). In the adult *L. pipiens*, atrazine may have caused respiratory distress due to an increase in buccal and thoracic ventilation. Yet no research has been conducted on reptilian life history or physiology.

In order to accurately assess the full range of effects of endocrine disruptors on both an individual and a population, the reproductive fitness of the exposed individual needs to be further quantified. Hormones control the development not only of the sex organs, but also of the brain. These neural components of development are related to the sexual dimorphism of brain nuclei, particularly the anteroventral periventricular nucleus and an area of the preoptic area. These areas are different in size between sexes, which is likely indicates a difference in function (Gore 2008).

When analyzing the neonates after exposure *in utero* to atrazine, several variables were not accounted for within this study. For example, while the neonates had the same genetic material from the mother, multiple paternity frequently occurs in natricine snakes (King et al. 2001; Prosser et al. 2002). Although the snakes were introduced to known

males in captivity, the females could have either stored sperm from previous years (as hypothesized in Prosser et al. 2002) and/or mated prior to capture.

One of the greatest challenges in evaluating the results reported here comes from the lack of knowledge of the normal functioning of reptilian endocrine systems. While some systems have been well studied (*e.g.*, Moore and Lindzey 1992; Whittier and Tokarz 1992), much remains unknown about production and control of hormones in reptiles. The difficulty in deciphering the subtleties of initial effects adds to the urgency and critical nature of this research. This study on the effects of atrazine on estradiol levels in gravid watersnakes and the subsequent development of the neonates is an important addition to the growing body of research addressing these questions.

REFERENCES

Abraxis LLC. 2008. 17β-Estradiol (E2) ELISA Kit (Microplate) User's guide. Warminster, PA.

Akaike H. 1974. A new look at the statistical model identification. IEEE Transactions on Automatic Control 19(6):716-723.

Ahmed SA. 2000. The immune system as a potential target for environmental estrogens (endocrine disruptors): A new emerging field. Toxicology 150(1-3):191-206.

Allran JW, Karasov WH. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environmental Toxicology and Chemistry 20(4):769-775.

Ames PL. 1966. DDT residues in the eggs of the osprey in the north-eastern United States and their relation to nesting success. Journal of Applied Ecology 3:87-97.

Barlow S, Kavlock RJ, Moore JA, Schantz SL, Sheehan DM, Shuey DL, Lary JM. 1999. Teratology society public affairs committee position paper: Developmental toxicity of endocrine disruptors to humans. Teratology 60(6):365-375.

Bergeron JM, Crews D, McLachlan JA. 1994. PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. Environmental Health Perspectives 102(9):780-781.

Bigsby R, Chapin RE, Daston GP, Davis BJ, Gorski J, Gray LE, Howdeshell KL, Zoeller RT, vom Saal FS. 1999. Evaluating the effects of endocrine disruptors on endocrine function during development. Environmental Health Perspectives 107(Suppl 4):613-618.

Biradar DP, Rayburn AL. 1995. Chromosomal damage induced by herbicide contamination at concentrations observed in public water supplies. Journal of Environmental Quality 24(6):1222-1225.

Blackburn DG. 2006. Squamate reptiles as model organisms for the evolution of viviparity. Herpetological Monographs 20(1):131-146.

Blackburn DG, Weaber KK, Stewart JR, Thompson MB. 2003. Do pregnant lizards resorb or abort inviable eggs and embryos? Morphological evidence from an Australian skink, *Pseudemoia pagenstecheri*. Journal of Morphology 256(2):219-234.

Bronson FH. 1981. The regulation of luteinizing hormone secretion by estrogen: Relationships among negative feedback, surge potential, and male stimulation in juvenile, peripubertal, and adult female mice. Endocrinology 108(2):506-516.

Brown GP, Weatherhead PJ. 1999. Demography and sexual size dimorphism in Northern Water Snakes, *Nerodia sipedon*. Canadian Journal of Zoology 77(9):1358-1366.

Brown GP, Weatherhead PJ. 2000. Thermal ecology and sexual size dimorphism in Northern Water Snakes, *Nerodia sipedon*. Ecological Monographs 70(2):311-330.

Brun GL, MacDonald RM, Verge J, Aubé J. 2008. Long-term atmospheric deposition of current-use and banned pesticides in Atlantic Canada; 1980-2000. Chemosphere 71(2):314-327.

Burger J. 2001. The behavioral response of basking Northern water (*Nerodia sipedon*) and Eastern garter (*Thamnophis sirtalis*) snakes to pedestrians in a New Jersey park. Urban Ecosystems 5(2):119-129.

Calabrese EJ. 2001. Estrogen and related compounds: Biphasic dose responses. Critical Reviews in Toxicology 31(4-5):503-515.

Chapman RN, Stranger JW. 1995. Horticultural pesticide residues in water-a survey of pesticide residues conducted in Gippsland, Victoria, 1994. Victorian Department of Agriculture (Australia).

Cheek AO, Brouwer TH, Carroll S, Manning S, McLachlan JA, Brouwer M. 2001. Experimental evaluation of vitellogenin as a predictive biomarker for reproductive disruption. Environmental Health Perspectives 109(7):681-690.

Christin MS, Menard L, Gendron AD, Ruby S, Cyr D, Marcogliese DJ, Rollins-Smith L, Fournier M. 2004. Effects of agricultural pesticides on the immune system of *Xenopus laevis* and *Rana pipiens*. Aquatic Toxicology 67(1):33-43.

Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA, Solomon KR, Smith EE, Van Der Kraak G, Kendall RJ, Giesy JP. 2005. Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. Ecotoxicology and Environmental Safety 62(2):160-173.

Colborn T. 2002. Clues from wildlife to create an assay for thyroid system disruption. Environmental Health Perspectives 110(Suppl 3):363-367.

Colborn T, Thayer K. 2000. Aquatic ecosystems: Harbingers of endocrine disruption. Ecological Applications 10(4):949-957.

Comber SDW. 1999. Abiotic persistence of atrazine and simazine in water. Pest Management Science 55(7):696-702.

Conant R, Collins JT. 1991. A Field Guide to Reptiles and Amphibians of Eastern and Central North America, 2nd ed. New York: Houghton Mifflin.

Coon NC, Locke LN, Cromartie E, Reichel WL. 1970. Causes of Bald Eagle mortality, 1960-1965. Journal of Wildlife Diseases 6(1):72-76.

Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK. 2000. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. Toxicological Sciences 53(2):297-307.

Crews D, Willingham E, Skipper JK. 2000. Endocrine disruptors: Present issues, future directions. Quarterly Review of Biology 75(3):243-260.

Cummings AM, Rhodes BE, Cooper RL. 2000. Effect of atrazine on implantation and early pregnancy in 4 strains of rats. Toxicological Sciences 58(1):135-143.

Danzo BJ. 1997. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environmental Health Perspectives 105(3):294-301.

Daston GP, Cook JC, Kavlock RJ. 2003. Uncertainties for endocrine disrupters: Our view on progress. Toxicological Sciences 74(2):245-252.

Davidson C, Benard MF, Shaffer HB, Parker JM, O'Leary C, Conlon JM, Rollins-Smith LA. 2007. Effects of Chytrid and carbaryl exposure on survival, growth, and skin peptide defenses in foothill yellow-legged frogs. Environmental Science and Technology 41(5):1771-1776.

De Solla SR, Bishop CA, Lickers H, Jock K. 2001. Organochlorine pesticides, PCBs, dibenzodioxin, and furan concentrations in Common Snapping Turtle eggs (*Chelydra serpentina serpentina)* in Akwesasne, Mohawk Territory, Ontario,

Canada. Archives of Environmental Contamination and Toxicology 40(3):410-417.

De Solla SR, Bishop CA, Van Der Kraak G, Brooks RJ. 1998. Impact of organochlorine contamination on levels of sex hormones and external morphology of Common Snapping Turtles (*Chelydra serpentina serpentina*) in Ontario, Canada. Environmental Health Perspectives 106(5):253-260.

De Solla SR, Fernie KJ. 2004. Characterization of contaminants in snapping turtles (*Chelydra serpentina*) from Canadian Lake Erie areas of concern: St. Clair River, Detroit River, and Wheatley Harbour. Environmental Pollution 132:101-112.

De Solla SR, Martin PA, Fernie KJ, Park BJ, Mayne G. 2006. Effects of environmentally relevant concentrations of atrazine on gonadal development of snapping turtles (*Chelydra serpentina*). Environmental Toxicology and Chemistry 25(2):520-526.

Du Preez HH, Van Vuren JHJ. 1992. Bioconcentration of atrazine in the banded tilapia, *Tilapia sparrmanii*. Comparative Biochemistry and Physiology 101(3):651-655.

Du Preez LH, Kunene N, Everson GJ, Carr JA, Giesy JP, Gross TS, Hosmer AJ, Kendall RJ, Smith EE, Solomon KR. 2008. Reproduction, larval growth, and reproductive development in African clawed frogs (*Xenopus laevis*) exposed to atrazine. Chemosphere 71(3):546-552.

Edginton AN, Rouleau C. 2005. Toxicokinetics of 14C-atrazine and its metabolites in stage-66 *Xenopus laevis*. Environmental Science and Technology 39(20):8083-8089.

Elsey RM, Lance VA, Joanen T, McNease L. 1991. Acute stress suppresses plasma estradiol levels in female alligators (*Alligator mississippiensis*). Comparative Biochemistry and Physiology Part A: Physiology 100(3):649-651.

Facemire CF, Gross TS, Guillette Jr LJ. 1995. Reproductive impairment of the Florida Panther: Nature or nurture? Environmental Health Perspectives 103(Suppl 4):79-86.

Fan W, Yanase T, Morinaga H, Gondo S, Okabe T, Nomura M, Hayes TB, Takayanagi R, Nawata H. 2007a. Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promoter II. Biochemical and Biophysical Research Communications 355(4):1012-1018.

Fan W, Yanase T, Morinaga H, Gondo S, Okabe T, Nomura M, Komatsu T, Morohashi K-I, Hayes TB, Takayanagi R, Nawata H. 2007b. Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans. Environmental Health Perspectives 115(5):720-727.

Fitch HS. 1960. Criteria for determining sex and breeding maturity in snakes. Herpetologica 16(1):49-51.

Fitch HS. 1987. Collecting and life-history techniques. In: Seigel RA, Collins JT, Novak SS, editors. Snakes: Ecology and Evolutionary Biology. New York: McGraw-Hill. pp 143-164.

Fraker MA. 1970. Home range and homing in the watersnake, *Natrix sipedon sipedon*. Copeia 1970(4):665-673.

Fry DM, Toone CK. 1981. DDT-induced feminization of gull embryos. Science 213(4510):922.

Fuerst EP, Norman MA. 1991. Interactions of herbicides with photosynthetic electron transport. Weed Science 39(3):458-464.

Gammon DW, Aldous CN, Carr WC Jr., Sanborn JR, Pfeifer KF. 2005. A risk assessment of atrazine use in California: Human health and ecological aspects. Pest Management Science 61(4):331-355.

Ganser LR, Hopkins WA, Neil L, Hasse S, Roe JH, Sever DM. 2003. Liver histopathology of the Southern Watersnake, *Nerodia fasciata fasciata*, following chronic exposure to trace element-contaminated prey from a coal ash disposal site. Journal of Herpetology 37(1):219-226.

Gibbons JW, Dorcas ME. 2004. North American Watersnakes: A Natural Hisotry. Norman, Okla: University of Oklahoma Press. 438 p.

Gill WB, Schumacher GF, Bibbo M, Straus 2nd FH, Schoenberg HW. 1979. Association of diethylstilbestrol exposure *in utero* with cryptorchidism, testicular hypoplasia and semen abnormalities. Journal of Urology 122(1):36-39.

Gioiosa L, Fissore E, Ghirardelli G, Parmigiani S, Palanza P. 2007. Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Hormones and Behavior 52(3):307-316.

Gore AC. 2008. Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. Frontiers in Neuroendocrinology 29(3):358-374.

Görge G, Nagel R. 1990. Kinetics and metabolism of 14C-lindane and 14C-atrazine in early life stages of zebrafish (*Brachydanio rerio*). Chemosphere 21(9):1125-1137.

Graymore M, Stagnitti F, Allinson G. 2001. Impacts of atrazine in aquatic ecosystems. Environment International 26(7-8):483-495.

Gregory PT, Larsen KW, Farr DR. 1992. Snake litter size = live young + dead young + yolks. Herpetological Journal 2:145-146.

Grier JW. 1982. Ban of DDT and subsequent recovery of reproduction in Bald Eagles. Science 218(4578):1232-1235.

Gross LM. 2009. Long-term assessment of predatory fish removal on a pond-breeding amphibian community in central Illinois. MS Thesis. Charleston: Eastern Illinois University.

Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environmental Health Perspectives 102:680-688.

Guillette LJ, Pickford DB, Crain DA, Rooney AA, Percival HF. 1996. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. General and Comparative Endocrinology 101(1):32-42.

Gunderson MP, Kools SAE, Milnes MR, Guillette LJ Jr. 2003. Effects of acute stress on plasma β-corticosterone, estradiol-17β and testosterone concentrations in juvenile American Alligators collected from three sites within the Kissimmee-Everglades

drainage basin in Florida (USA). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 135(3):365-374.

Haavisto T, Nurmela K, Pohjanvirta R, Huuskonen H, El-Gehani F, Paranko J. 2001. Prenatal testosterone and luteinizing hormone levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol. Molecular and Cellular Endocrinology 178(1-2):169-179.

Hadley ME, Levine JE. 2003. Endocrinology. San Francisco: Benjamin Cummins. 608 p.

Hatfield JL, Wesley CK, Prueger JH, Pfeiffer RL. 1996. Herbicide and nitrate distribution in Central Iowa rainfall. Journal of Environmenal Quality 25(2):259-264.

Hayes TB. 2004. There is no denying this: Defusing the confusion about atrazine. BioScience 54(12):1138-1149.

Hayes TB. 2005. Welcome to the revolution: Integrative biology and assessing the impact of endocrine disruptors on environmental and public health. Integrative and Comparative Biology 45(2):321-329.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA. 2002a. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Science: USA 99(8):5476-5480.

Hayes TB, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2002b. Herbicides: Feminization of male frogs in the wild. Nature 419:1.

Hayes TB, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in Leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environmental Health Perspectives 111(4):568-575.

Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, Adame L, Chan E, Buchholz D, Stueve T, Gallipeau S. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Science: USA 107(10):4612-4617.

Hayes TB, Stuart AA, Mendoza M, Collins A, Noriega N, Vonk A, Johnston G, Liu R, Kpodzo D. 2006. Characterization of atrazine-induced gonadal malformations in African Clawed Frogs (Xenopus laevis) and comparisons with effects of an androgen antagonist (Cyproterone Acetate) and exogenous estrogen (17β-Estradiol): Support for the demasculinization/feminization hypothesis. Environmental Health Perspectives 114(1):134-141.

Hecker M, Park J-W, Murphy MB, Jones PD, Solomon KR, Van Der Kraak G, Carr JA, Smith EE, du Preez L, Kendall RJ, Giesy JP. 2005. Effects of atrazine on CYP19 gene expression and aromatase activity in testes and on plasma sex steroid concentrations of male African Clawed Frogs (*Xenopus laevis*). Toxicological Sciences 86(2):273-280.

Heindel JJ. 2003. Endocrine disruptors and the obesity epidemic. Toxicological Sciences 76(2):247-249.

Hopkins WA. 2000. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. Environmental Toxicology and Chemistry 19(10):2391-2393.

Hopkins WA, Roe JH, Snodgrass JW, Jackson BP, Kling DE, Rowe CL, Congdon JD. 2001. Nondestructive indices of trace element exposure in squamates reptiles. Environmental Pollution 115(1)1-7.

Hopkins WA, Roe JH, Snodgrass JW, Staub BP, Jackson BP, Congdon JD. 2002. Effects of chronic dietary exposure to trace elements on banded water snakes (*Nerodia fasciata*). Environmental Toxicology and Chemistry 21(5):906-913.

Hopkins WA, Rowe CL, Congdon JD. 1999. Elevated trace element concentrations and standard metabolic rate in Banded Watersnakes (*Nerodia fasciata*) exposed to coal combustion wastes. Environmental Toxicology and Chemistry 18(6):1258-1263.

Hopkins WA, Staub BP, Baionno JA, Jackson BP, Talent LG. 2005. Transfer of selenium from prey to predators in a simulated terrestrial food chain. Environmental Pollution 134(3):447-456.

Hoppmann E, Barron HW. 2007. Dermatology in reptiles. Journal of Exotic Pet Medicine 16(4):210-224.

Jemison SC, Bishop LA, May PG, Farrell TM. 1995. The impact of PIT-tags on growth and movement of the rattlesnake, *Sistrurus miliarius*. Journal of Herpetology 29(1):129-132.

Jooste AM, Du Preez LH, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Van Der Kraak GL, Solomon KR. 2005. Gonadal development of larval male *Xenopus*

laevis exposed to atrazine in outdoor microcosms. Environmental Science & Technology 39(14):5255-5261.

Kalkhoff SJ, Barnes KK, Becher KD, Savoca ME, Schnoebelen DJ, Sadorf EM, Porter SD, Sullivan DJ. 2000. Water Quality in the Eastern Iowa Basins, Iowa and Minnesota, 1996–98: U.S. Geological Survey Circular Survey. U.S. Geological Survey.

Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S, Lucier G, Luster M, Mac MJ, Maczka C. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the US EPA-sponsored workshop. Environmental Health Perspectives 104(Suppl 4):715-740.

King RB. 1987. Color pattern polymorphism in the Lake Erie water snake, *Nerodia sipedon insularum*. Ecology 41(2):241-255.

King RB. 1993. Microgeographic, historical, and size-correlated variation in water snake diet composition. Journal of Herpetology 27(1):90-94.

King RB, Milstead WB, Gibbs HL, Prosser MR, Burghardt GM, and McCracken GF. 2001. Application of microsatellite DNA markers to discriminate between maternal and genetic effects on scalation and behavior in multiply-sired garter snake litters. Canadian Journal of Zoology 79:121-128.

Koller LD, Kovacic S. 1974. Decreased antibody formation in mice exposed to lead. Nature 250(5462):148-150.

Komang RT, Senior E, van Verseveld HW. 2002. Microbial aspects of atrazine degradation in natural environments. Biodegradation 13(1):11-19.

Kruger KL, Somasundaram L, Coats JR, Kanwar RS. 1993. Persistence and degradation of [14C]atrazine and [14C]deisopropylatrazine as affected by soil depth and moisture conditions. Environmental Health Perspectives 12(11):1959-1967.

Landrigan PJ, Claudio L, Markowitz SB, Berkowitz GS, Brenner BL, Romero H, Wetmur JG, Matte TD, Gore AC, Godbold JH. 1999. Pesticides and inner-city children: Exposures, risks, and prevention. Environmental Health Perspectives 107(Suppl 3):431.

Langlois VS, Carew AC, Pauli BD, Wade MG, Cooke GM, Trudeau VL. 2009. Low levels of the herbicide atrazine alter sex ratios and reduce metamorphic success in *Rana pipiens* tadpoles raised in outdoor mesocosms. Environmental Health Perspectives 118(4):552-557.

Marra PP, Lampe KT, Tedford BL. 1995. Plasma corticosterone levels in two species of *Zonotrichia* sparrows under captive and free-living conditions. Wilson Bulletin 107(2):296-305.

Mast M, Foreman W, Skaates S. 2007. Current-use pesticides and organochlorine compounds in precipitation and lake sediment from two high-elevation national parks in the western United States. Archives of Environmental Contamination and Toxicology 52(3):294-305.

Mathies T, Felix TA, Lance VA. 2001. Effects of trapping and subsequent short-term confinement stress on plasma corticosterone in the Brown Treesnake (*Boiga irregularis*) on Guam. General and Comparative Endocrinology 124(1):106-114.

Matsushita S, Yamashita J, Iwasawa T, Tomita T, Ikeda M. 2006. Effects of *in ovo* exposure to imazalil and atrazine on sexual differentiation in chick gonads. Poultry Science 85(9):1641-1647.

McCarthy ID, Fuiman LA. 2008. Growth and protein metabolism in Red Drum (*Sciaenops ocellatus*) larvae exposed to environmental levels of atrazine and malathion. Aquatic Toxicology 88(4):220-229.

McMullin TS, Andersen ME, Nagahara A, Lund TD, Pak T, Handa RJ, Hanneman WH. 2004. Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-Dawley Rats without changing estrogen receptor action. Toxicological Sciences 79:278-286.

Moore IT, Greene MJ, Lerner DT, Asher CE, Krohmer RW, Hess DL, Whittier J, Mason RT. 2005. Physiological evidence for reproductive suppression in the introduced population of brown tree snakes (*Boiga irregularis*) on Guam. Biological Conservation 121(1):91-98.

Moore IT, Jessop TS. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Hormones and Behavior 43(1):39-47.

Moore IT, Lerner JP, Lerner DT, Mason RT. 2000. Relationships between annual cycles of testosterone, corticosterone, and body condition in male red-spotted garter snakes, *Thamnophis sirtalis concinnus*. Physiological and Biochemical Zoology 73(3):307-312.

Moore MC, Lindzey J. 1992. Physiological basis of sexual behavior in male reptiles. In: Biology of the Reptilia, vol 18 Horomones, Brain, and Behavior. Gans C, Crews D, editors. Chicago: University of Chicago Press. pp 70-113.

Morse SI, Stearns CD, Goldsmith SR. 1975. Lymphocyte depletion induced by Cholera toxin; relationship to adrenal cortical function. Journal of Immunology 114(2):665-670.

Munkittrick KR, Servos MR, Van Der Kraak GJ, McMaster ME, Portt CB, Van Den Heuvel MR. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills: 2. Gonad size, liver size, hepatic erod activity and plasma sex steroid levels in white sucker. Environmental Toxicology and Chemistry 13(7):1089-1101.

Murray SM, Gaines KF, Novak JM, Gochfeld M, Burger J. 2010. DNA double-strand breakage as an endpoint to examine metal and radionuclide exposure effects to water snakes on a nuclear industrial site. Human and Ecological Risk Assessment 16(2):282-300.

Mushinsky HR. 1979. Mating behavior of the Common Water Snake, *Nerodia sipedon sipedon*, in Eastern Pennsylvania (Reptilia, Serpentes, Colubridae). Journal of Herpetology 13(1):127-129.

Neuman-Lee L, Janzen FJ. 2010. Environmentally relevant levels of atrazine exposure at the onset of embryonic development impacts post-hatching behavior and survivorship of neonatal turtles. Herpetologica 66:In press.

Newman AEM, Chin EH, Schmidt KL, Bond L, Wynne-Edwards KE, Soma KK. 2008. Analysis of steroids in songbird plasma and brain by coupling solid phase

extraction to radioimmunoassay. General and Comparative Endocrinology 155(3):503-510.

Newman MC, Unger MA. 2003. Fundamentals of Toxicology. Boca Raton: CRC Press LLC.

Ohlendorf HM, Hothem RL, Aldrich TW. 1988. Bioaccumulation of selenium by snakes and frogs in the San Joaquin Valley, California. Copeia 1988(3):704-710.

Olds MJ. 2007. Habitat use and overwintering ecology of the Northern Watersnake (*Nerodia sipedon*) on an artificial levee in central Illinois. M.S. Thesis. Charleston: Eastern Illinois University.

Pimentel D, Acquay H, Biltonen M, Rice P, Silva M, Nelson J, Lipner V, Giordano S, Horowitz A, D'Amore M. 1992. Environmental and economic costs of pesticide use. BioScience 42(10):750-760.

Propper C. 2005. The study of endocrine-disrupting compounds: Past approaches and new directions. Integrative and Comparative Biology 45(6):194-200.

Prosser MR, Weatherhead PJ, Gibbs HL, Brown GP. 2002. Genetic analysis of the mating system and opportunity for sexual selection in Northern Water Snakes (*Nerodia sipedon*). Behavioral Ecology 13(6):800-807.

Pruett SB, Fan R, Zheng Q, Myers LP, Hebert P. 2003. Modeling and predicting immunological effects of chemical stressors: Characterization of a quantitative biomarker for immunological changes caused by atrazine and ethanol. Toxicological Sciences 75(2):343-354.

Ratcliffe DA. 1970. Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some British birds. Journal of Applied Ecology 7(1):67-115.

Riches RJ. 1976. Breeding snakes in captivity. St. Petersburg: Palmetto Publishing Company.

Rivas A, Fisher JS, McKinnell C, Atanassova N, Sharpe RM. 2002. Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: Evidence for importance of the androgen-estrogen balance. Endocrinology 143(12):4797-4808.

Robert KA, Vleck C, Bronikowski AM. 2009. The effects of maternal corticosterone levels on offspring behavior in fast- and slow-growth garter snakes (*Thamnophis elegans*). Hormones and Behavior 55(1):24-32.

Rodriguez VM, Thiruchelvam M, Cory-Slechta DA. 2005. Sustained exposure to the widely used herbicide atrazine: Altered function and loss of neurons in brain monoamine systems. Environmental Health Perspectives 113(6):708-715.

Rohr JR, McCoy KA. 2010. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environmental Health Perspectives 118(1):20-32.

Rohr JR, Sager T, Sesterhenn TM, Palmer BD. 2006. Exposure, postexposure, and density-mediated effects of atrazine on amphibians: Breaking down net effects into their parts. Environmental Health Perspectives 114(1):46-50.

Sanderson JT. 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Environmental Health Perspectives 94(1):3-21.

Sass JB, Colangelo A. 2006. European Union bans atrazine, while the United States negotiates continued use. International Journal of Occupational and Environmental Health 12(3):260-267.

Scudder KM, Stewart NJ, Smith HM. 1980. Response of neonate water snakes (*Nerodia sipedon sipedon*) to conspecific chemical cues. Journal of Herpetology 14(2):196-198.

Seigel RA, Fitch HS. 1984. Ecological patterns of relative clutch mass in snakes. Oecologia 61:293-301.

Sharpe RM. 2006. Pathways of endocrine disruption during male sexual differentiation and masculinisation. Best Practice & Research Clinical Endocrinology & Metabolism 20(1):91-110.

Sharpe RM, Skakkebaek NE. 2003. Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research. Pure and Applied Chemistry 75(11-12):2023-2038.

Sigma-Aldrich Co. 1998. Guide to Solid Phase Extraction. St. Louis, Missouri.

Snyder MJ, Mulder EP. 2001. Environmental endocrine disruption in decapods crustacean larvae: Hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. Aquatic Toxicology 55(3-4):177-190.

Solomon KR, Baker DB, Richards RP, Dixon KR, Klaine SJ, La Point TW, Kendall RJ, Weisskopf CP, Giddings JM, Giesy JP, Hall LW, Williams WM. 1996. Ecological risk assessment of atrazine in North American surface waters. Environmental Toxicology and Chemistry 15(1):31-76.

Solomon KR, Carr JA, Du Preez LH, Giesy JP, Kendall RJ, Smith EE, Van Der Kraak GJ. 2008. Effects of atrazine on fish, amphibians, and aquatic reptiles: A critical review. Critical Reviews in Toxicology 38(9):721-772.

Spanò L, Tyler CR, van Aerle R, Devos P, Mandiki SNM, Silvestre F, Thomé JP, Kestemont P. 2004. Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). Aquatic Toxicology 66(4):369-379.

Stebbing ARD. 2003. A mechanism for hormesis--A problem in the wrong discipline. Critical Reviews in Toxicology 33(3&4):463-467.

Stickel LF, Wiemeyer SN, Blus LJ. 1973. Pesticide residues in eggs of wild birds: Adjustment for loss of moisture and lipid. Bulletin of Environmental Contamination and Toxicology 9(4):193-196.

Stillman RJ. 1982. *In utero* exposure to diethylstilbestrol: Adverse effects on the reproductive tract and reproductive performance and male and female offspring. American Journal of Obstetrics and Gynecology 142(7):905-921.

Stoker TE, Laws SC, Guidici DL, Cooper RL. 2000. The effect of atrazine on puberty in male Wistar rats: An evaluation in the protocol for the assessment of pubertal development and thyroid function. Toxicological Sciences 58(1):50-59.

Storrs SI, Kiesecker JM. 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environmental Health Perspectives 112(10):3.

Sumpter JP, Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives 103(Suppl 7):173-178.

Tabb MM, Blumberg B. 2006. New modes of action for endocrine-disrupting chemicals. Molecular Endocrinology 20(3):475-482.

Taylor EN, DeNardo DF, Jennings DH. 2004. Seasonal steroid hormone levels and their relation to reproduction in the Western Diamond-backed Rattlesnake, *Crotalus atrox* (Serpentes: Viperidae). General and Comparative Endocrinology 136(3):328-337.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette Jr LJ, Jegou B, Jensen TK, Jouannet P, Keiding N. 1996. Male reproductive health and environmental xenoestrogens. Environmental Health Perspectives 104(Suppl 4):741-803.

Travis CC, Hattemer-Frey HA. 1988. Determining an acceptable level of risk. Environmental Science & Technology 22(8):873-876.

Újvári B, Madsen T, Kotenko T, Olsson M, Shine R, Wittzell H. 2002. Low genetic diversity threatens imminent extinction for the Hungarian meadow viper (*Vipera ursinii rakosiensis*). Biological Conservation 105(1):127-130.

USEPA (US Environmental Protection Agency). 2009. Atrazine science re-evaluation: Potential health risks. Available from <http://www.regulations.gov/search/Regs/home.html#documentDetail?R=0900006480a3dabc>

US Fish and Wildlife Service. 2010. Threatened and Endangered Species Database System. Department of the Interior. Available from <http://www.fws.gov/endangered/wildlife.html#Species>

vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV. 1997. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proceedings of the National Academy of Science: USA 94(5):2056-2061.

Vos JG, Moore JA. 1977. Immune suppression as related to toxicology. Critical Reviews in Toxicology 5(1):67-101.

Wassermann M, Wassermann D, Gershon Z, Zellermayer L. 1969. Effects of organochlorine insecticides on body defense systems. Annals of the New York Academy of Sciences 160(Biological Effects of Pesticides in Mammalian Systems):393-401.

Weatherhead PJ, Barry FE, Brown GP, Forbes MRL. 1995. Sex ratios, mating behavior and sexual size dimorphism of the northern water snake, *Nerodia sipedon*. Behavioral Ecology and Sociobiology 36(5):301-311.

Weatherhead PJ, Brown GP, Prosser MR, Kissner KJ. 1998. Variation in offspring sex ratios in the northern water snake (*Nerodia sipedon*). Journal of Zoology 76(12):2200-2206.

Weatherhead PJ, Brown GP, Prosser MR, Kissner KJ. 1999. Factors affecting neonate size variation in northern water snakes, Nerodia sipedon. Journal of Herpetology 33(4):577-589.

Weil MR, Aldridge RD. 1981. Seasonal androgenesis in the male water snake, *Nerodia sipedon*. General and Comparative Endocrinology 44(1):44-53.

Whittier JM, Mason RT, Crews D. 1987. Plasma steroid hormone levels of female red-sided garter snakes, *Thamnophis sirtalis parietalis*: relationship to mating and gestation. General and Comparative Endocrinology 67(1):33-43.

Whittier JM, Tokarz RR. 1992. Physiological regulation of sexual behavior in female reptiles. In: Biology of the Reptilia, vol. 18 Hormones, Brain, and Behavior. Gans C, Crews D, editors. Chicago: University of Chicago Press. pp 24-69.

Williams K, McKinnell C, Saunders PTK, Walker M, Fisher JS, Turner KJ, Atanassova N, Sharpe RM. 2001. Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: Evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. Human Reproduction Update 7(3):236-247.

Willingham EJ. 2005. The effects of atrazine and temperature on turtle hatchling size and sex ratios. Frontiers in Ecology and the Environment 3(6):309-313.

Winkelmann DA, Klaine SJ. 1991. Degradation and bound residue formation of four atrazine metabolites, deethylatrazine, deisopropylatrazine, dealkylatrazine and hydroxyatrazine, in a western Tennessee soil. Environmental Toxicology and Chemistry 10(3):347-354.

Winne CT, Willson JD, Andrews KM, Reed RN. 2006. Efficacy of marking snakes with disposable medical cautery units. Herpetology Review 37(1):52-54.

Table 1. Mean (±1 standard error), minimum, and maximum snout-vent length (mm), initial mass (g) at capture, and final mass (after 3 months of ingestion of atrazine) for female *Nerodia sipedon* caught in central Illinois in 2009.

	Snout-Vent Length			Initial Mass			Final Mass		
Treatment	Mean	Minimum	Maximum	Mean	Minimum	Maximum	Mean	Minimum	Maximum
Control	844.9 ± 52.1	656	1005	526.4 ± 98.1	205	945	509.9 ± 96.0	181	819
2 ppb	884.0 ± 113.8	806	977	610.6 ± 65.3	375	869	565.0 ± 55.6	326	758
20 ppb	785.8 ± 22.9	690	850	408.9 ± 32.7	283	522	403.0 ± 29.9	302	487
200 ppb	845.3 ± 37.0	727	925	521.8 ± 79.7	248	712	546.2 ± 69.8	299	714
Overall	813.7 ± 33.8	656	1005	516.9 ± 38.0	205	945	506.0 ± 34.8	181	819

Table 2. Akaike information criteria (AIC) values determining the best fit for the nonlinear model for the concentration of estradiol in female Northern Watersnakes (*Nerodia sipedon*). ΔAIC is the difference between the best fit model (lowest AIC) and the AIC value for that parameter. w_i is calculated by taking the negative square-root of the ΔAIC. That value is divided by the next best parameter to determine the evidence ratio.

Model	# of Parameters	AIC	ΔAIC	w_i	Evidence ratio
Quartic	5	2397.47	0.00	0.64	2.00
Quintic	6	2398.86	1.39	0.32	2.72
Sextic	7	2400.86	3.39	0.12	44.26
Quadratic	3	2408.44	10.97	0.002	1.02
Linear	2	2408.48	11.01	0.003	1.67
Cubic	4	2409.50	12.03	0.002	--

Table 3. Causes of death described for gravid Northern Watersnakes (*Nerodia sipedon*) exposed to different concentrations of atrazine during gestation in 2009, with number of individuals dying within each treatment.

Cause of death	Treatment	Number affected
Infection (see text)	Control	1
	2 ppb	3
	20 ppb	2
	200 ppb	3
Oviduct perforated by calcified embryonic plug	Control	1
Tumor	Control	1
Intestinal blockage (individual displayed signs of advanced infection as well)	200 ppb	1
Unknown (individual had symptoms of advanced infection)	2 ppb	1

Table 4. Mean (±1 standard error) relative clutch masses of Northern Watersnake (*Nerodia sipedon*) clutches after ingesting atrazine throughout the gestation period (3 mo.) in 2009, as calculated by dividing the total clutch mass in grams (all embryos and neonates) by the post-birth weight of the female.

Treatment	n	Relative Clutch Mass (RCM)
Control	5	0.15 ± 0.03
2 ppb	5	0.13 ± 0.03
20 ppb	6	0.12 ± 0.02
200 ppb	2	0.11 ± 0.04
Total	18	0.13 ± 0.01

Table 5. Mean (±1 standard error), minimum, maximum values for snout-vent length (mm) and mass (g) of neonate Northern Watersnakes (*Nerodia sipedon*) after being exposed *in utero* to ingested atrazine throughout development.

		Neonate Snout-Vent Length			Neonate Mass		
Treatment	n	Mean	Minimum	Maximum	Mean	Minimum	Maximum
Control	5	164.20 ± 7.11	135.55	183.54	3.68 ± 0.36	2.43	4.94
2 ppb	5	161.41 ± 7.65	138.25	184.9	3.28 ± 0.35	2.22	4.58
20 ppb	6	153.57 ± 4.61	137.56	164.00	3.39 ± 0.65	2.01	6.21
200 ppb	2	161.05 ± 5.48	151.56	170.53	2.98 ± 0.13	2.74	3.20
Overall	18	160.1 ± 3.09			3.3 ± 0.22		

Table 6. Summary of the effects of atrazine ingestion during gestation on gravid Northern Watersnakes (*Nerodia sipedon*) and their subsequent offspring. [*Discussed in Appendix II.]

Endpoint	Effect of atrazine
Estradiol levels	Yes—atrazine exposure explained some variation in estradiol levels over the course of gestation.
Sex ratio	Yes—the 20 and 200 ppb exposure treatments had male-biased sex ratios.
Mortality	No
Cause of death	Yes—larger females exposed to atrazine were more likely to die from infection.
Stillbirths	Yes—females tended to birth more stillborn neonates following exposure to atrazine ($p = 0.08$).
Liver lipid mass in females	No*
Liver lipid in mass in neonates	No *

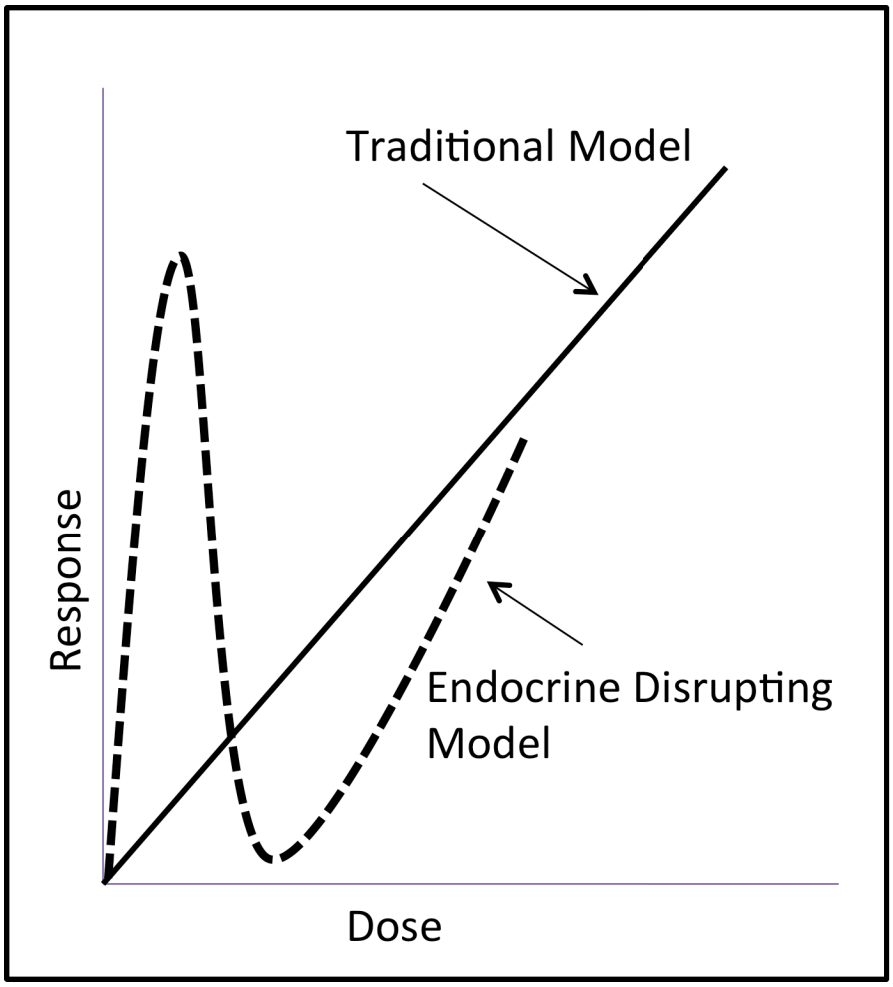

Figure 1. The traditional model versus an endocrine disrupting model of dose-response. With increasing dose, the response also increases in the traditional model. In the endocrine disrupting, or nonmonotonic, model the response is high at a low level before decreasing and increasing again (modified from Bigsby et al. 1999).

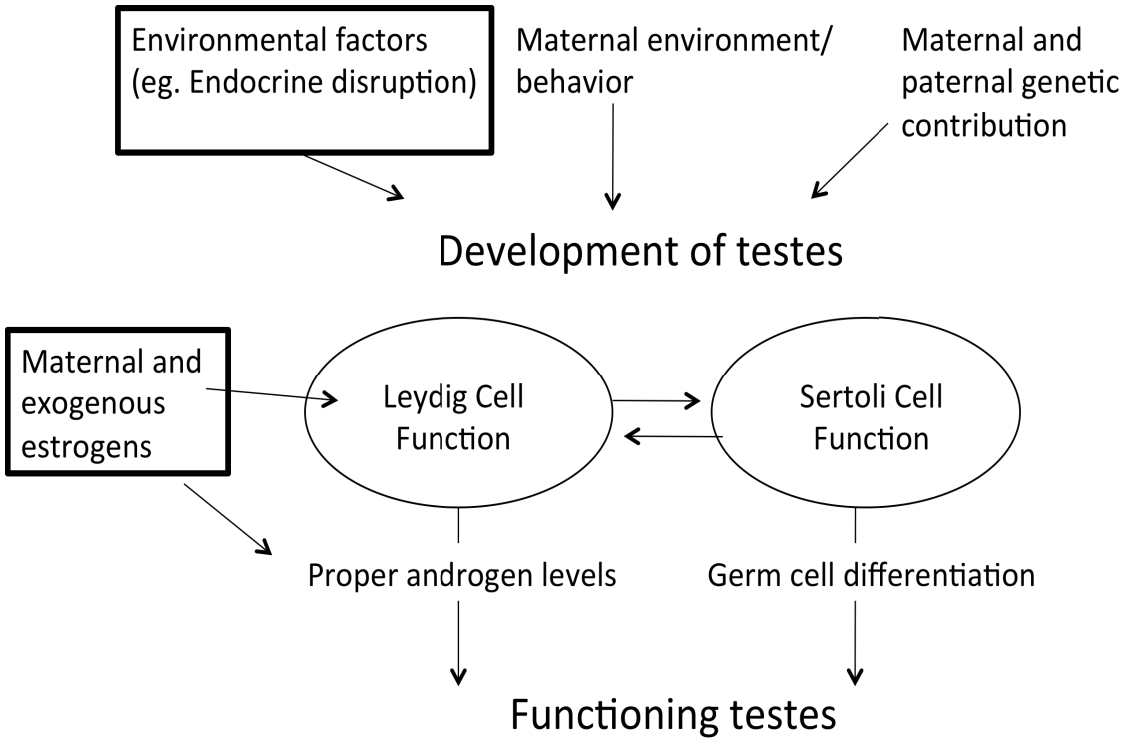

Figure 2. A diagram of the multiple factors influencing proper development of testes and the two aspects (environmental factors and maternal and exogenous estrogens) of potential maldevelopment examined in this thesis (modified from Sharpe and Skakkebaek 2003).

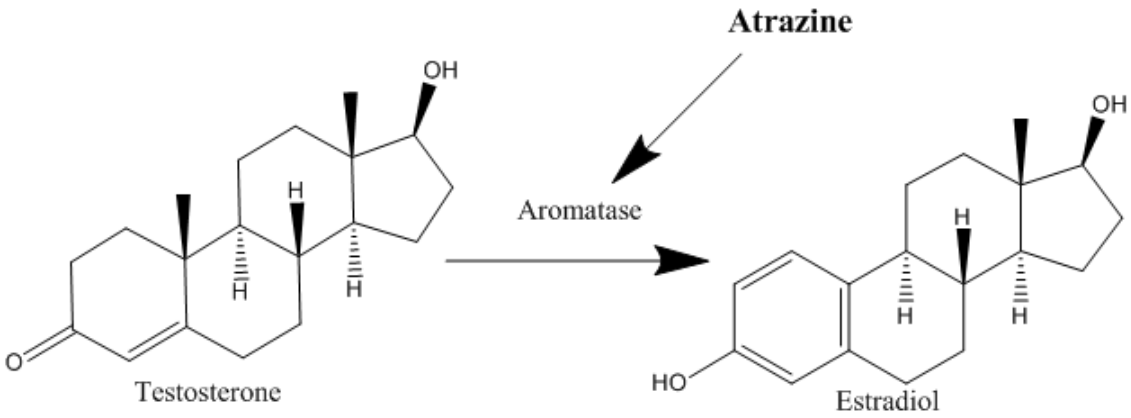

Figure 3. An illustration of the conversion of testosterone to estradiol in a typical vertebrate. One hypothesized mechanism of atrazine action is the excess induction of aromatase, therefore increasing the levels of estradiol in a system.

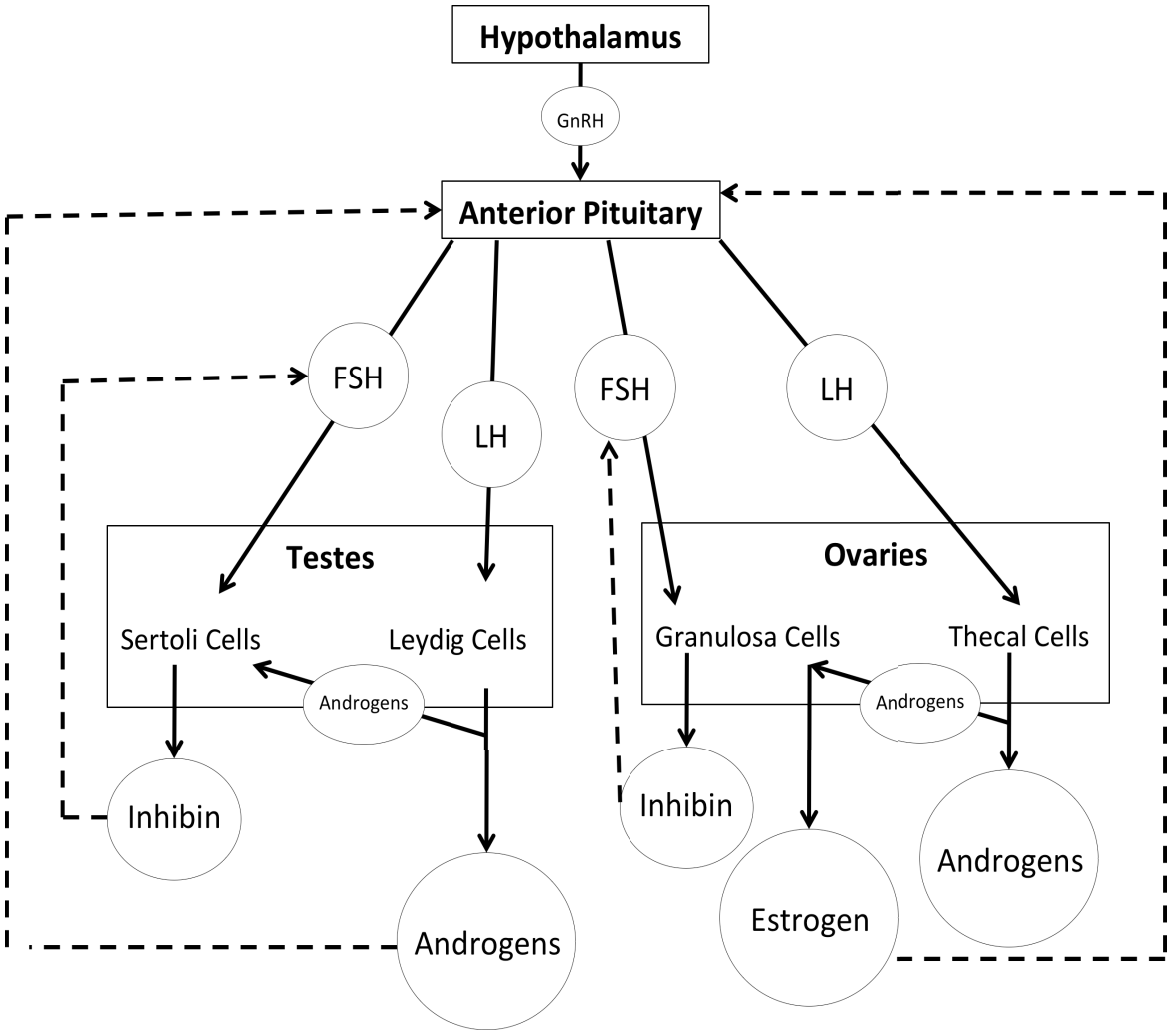

Figure 4. A diagram of the negative feedback loop of estrogen and androgens in the ovaries and testes (GnRH = Gonadotropin releasing hormones; FSH = Follicle stimulating hormone; LH = luteinizing hormone).

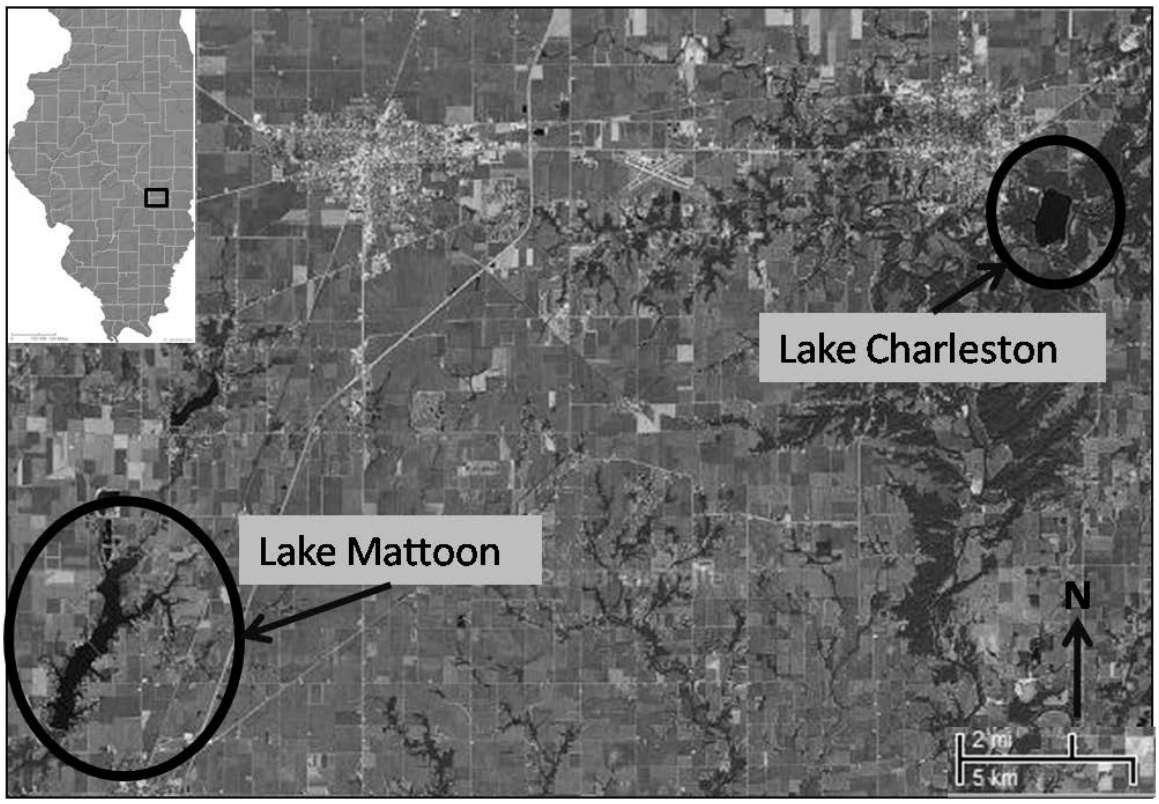

Figure 5. A satellite image of the locations and surrounding habitats of Lake Charleston and Lake Mattoon (in circles). The town to the northwest is Mattoon while the town directly northwest of Lake Charleston is Charleston, Illinois.

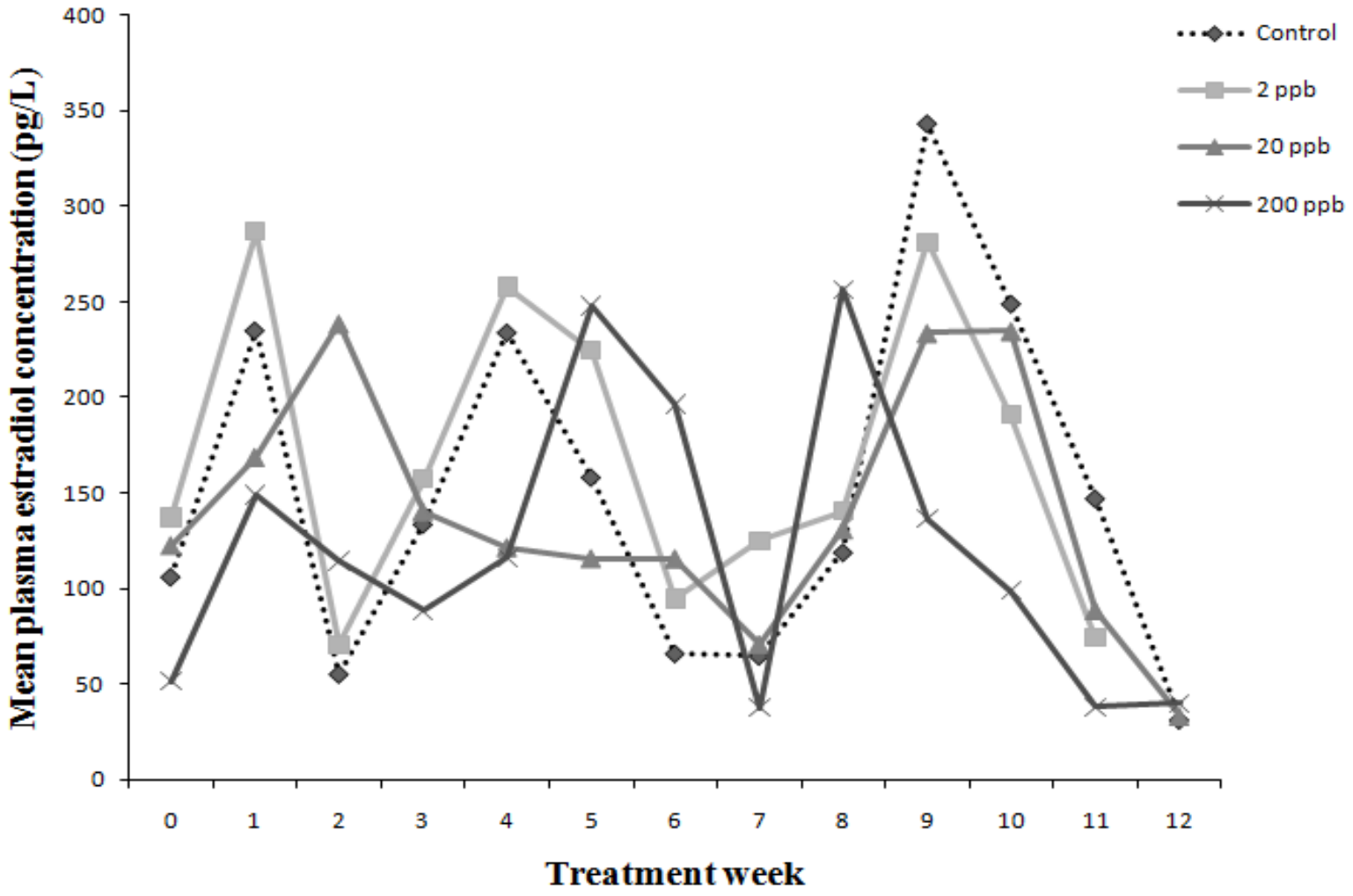

Figure 6. Mean plasma estradiol concentration in female Northern Watersnakes (*Nerodia sipedon*) exposed to one of four atrazine treatments as a function of the week of treatment.

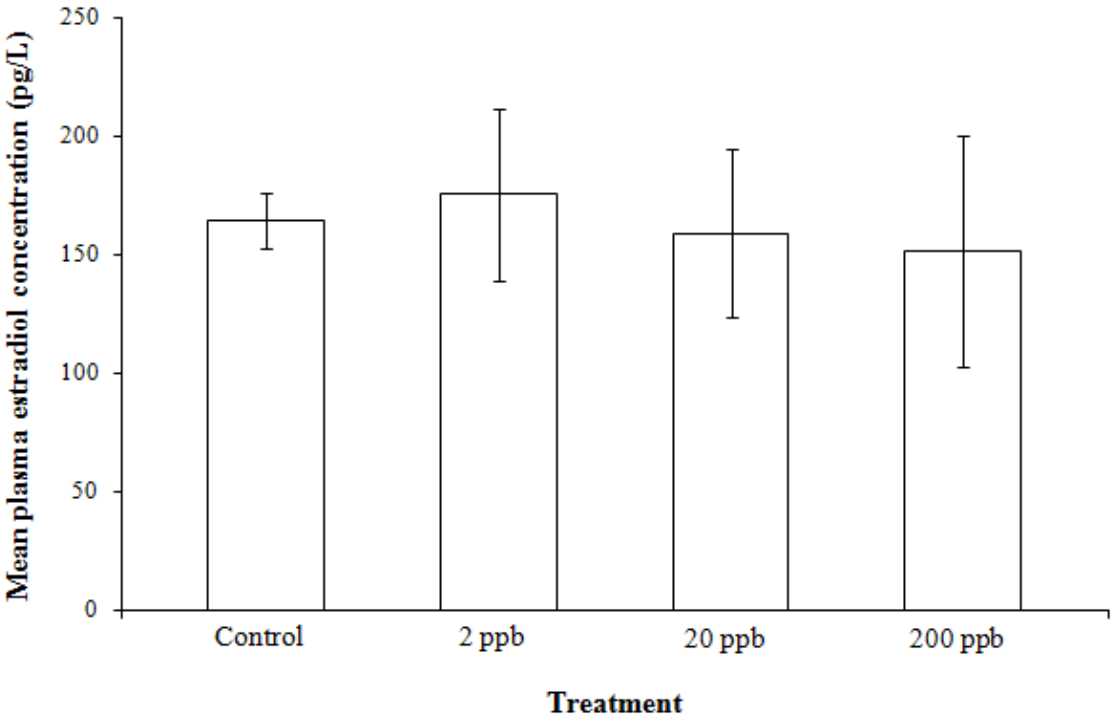

Figure 7. Mean estradiol concentration (± 95% confidence interval) in the plasma of gravid Northern Watersnakes (*Nerodia sipedon*) during 3 months of atrazine ingestion in 2009.

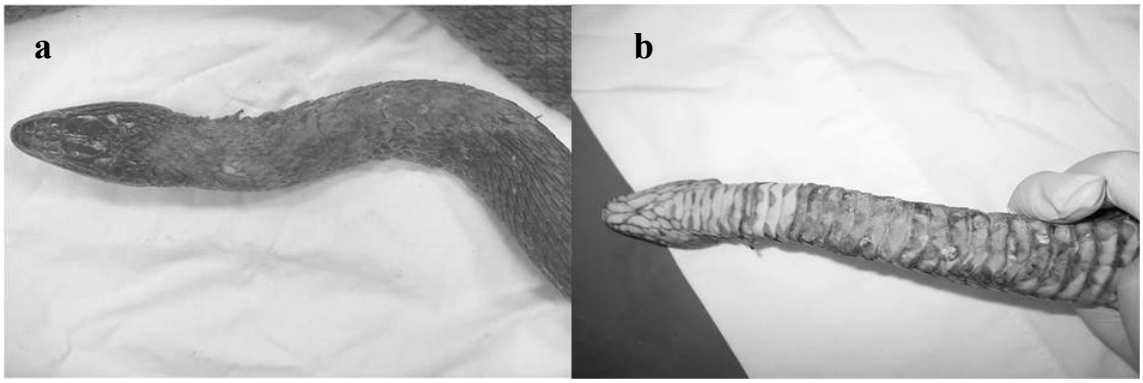

Figure 8. Dorsal (a) and ventral (b) views of an advanced stage of scale infection of an adult female Northern Watersnake (*Nerodia sipedon*) held in captivity for two and a half months. This individual died one week after photograph was taken.

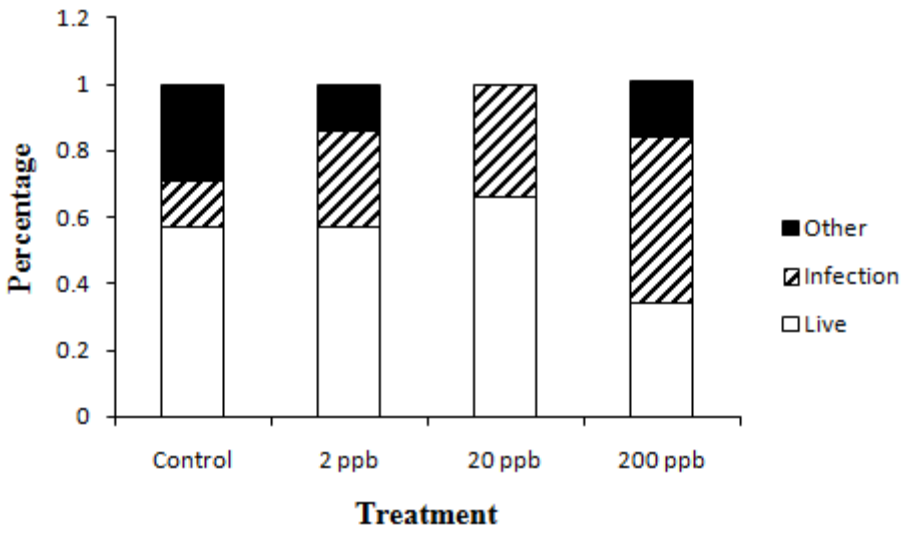

Figure 9. Fate (expressed as a percentage) of Northern Watersnakes (*Nerodia sipedon*) for each of four treatment groups expressed as a percentage (Control n = 7; 2 ppb n = 7; 20 ppb n = 6; 200 ppb n = 6).

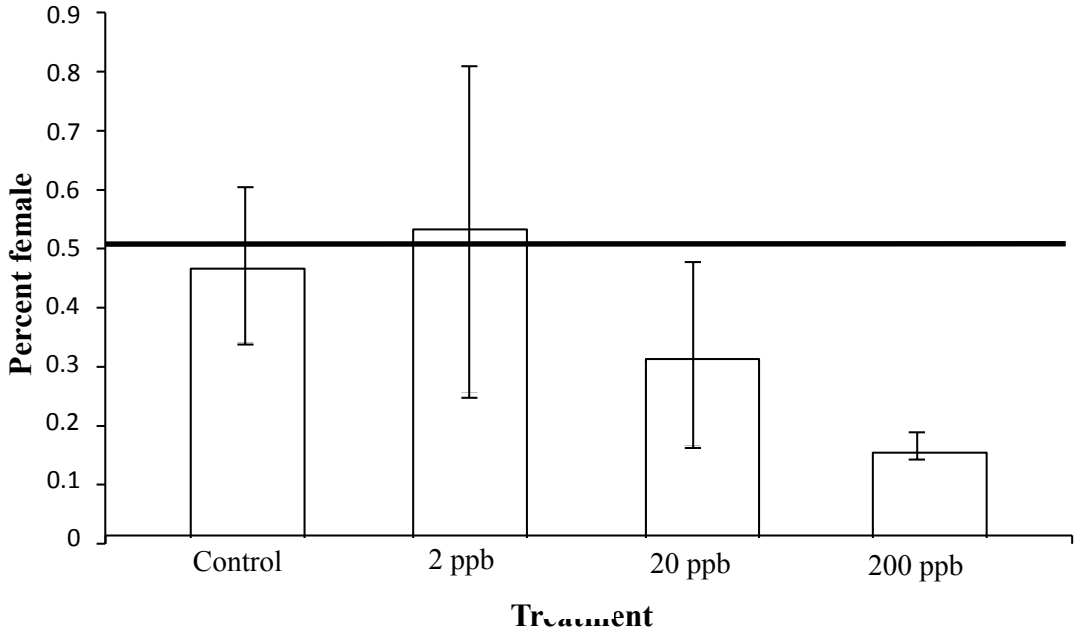

Figure 10. Percent female *Nerodia sipedon* neonates (± 95% confidence interval) born after being exposed to atrazine *in utero* mother snakes ingested atrazine-laced fish throughout their pregnancy. The black line indicates the expected 50% female neonates.

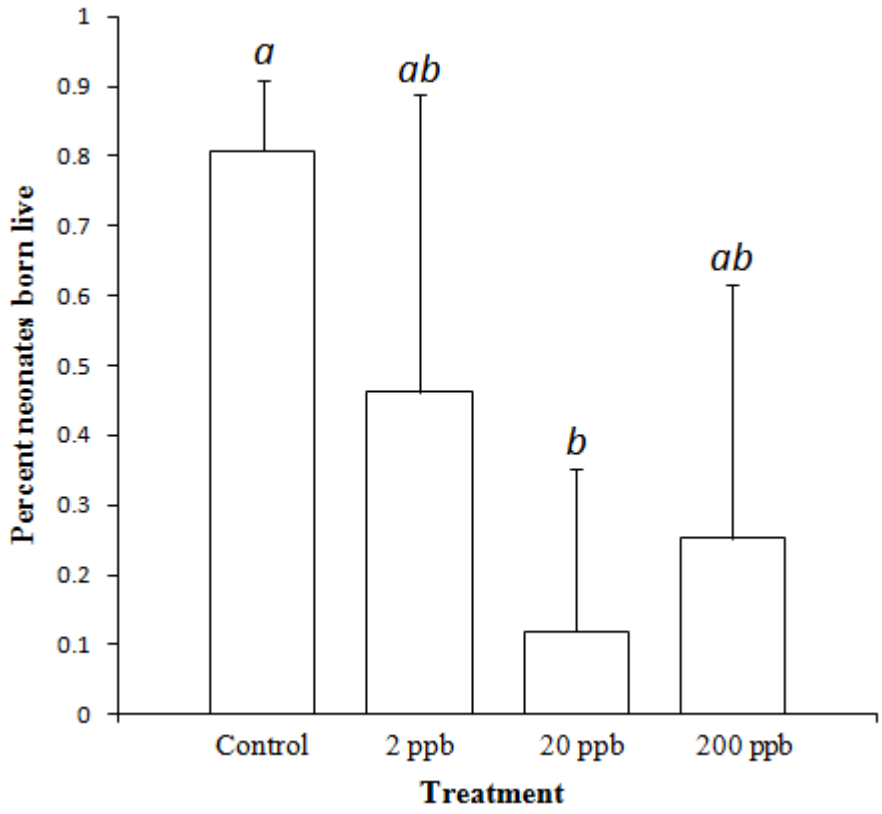

Figure 11. Mean percentage (+ 95% confidence interval) of neonate Northern Watersnakes (*Nerodia sipedon*) born live per treatment (Control n = 3; 2 ppb n = 4; 20 ppb n = 4; 200 ppb n = 2). Different superscripts above bars denote statistically-distinct values.

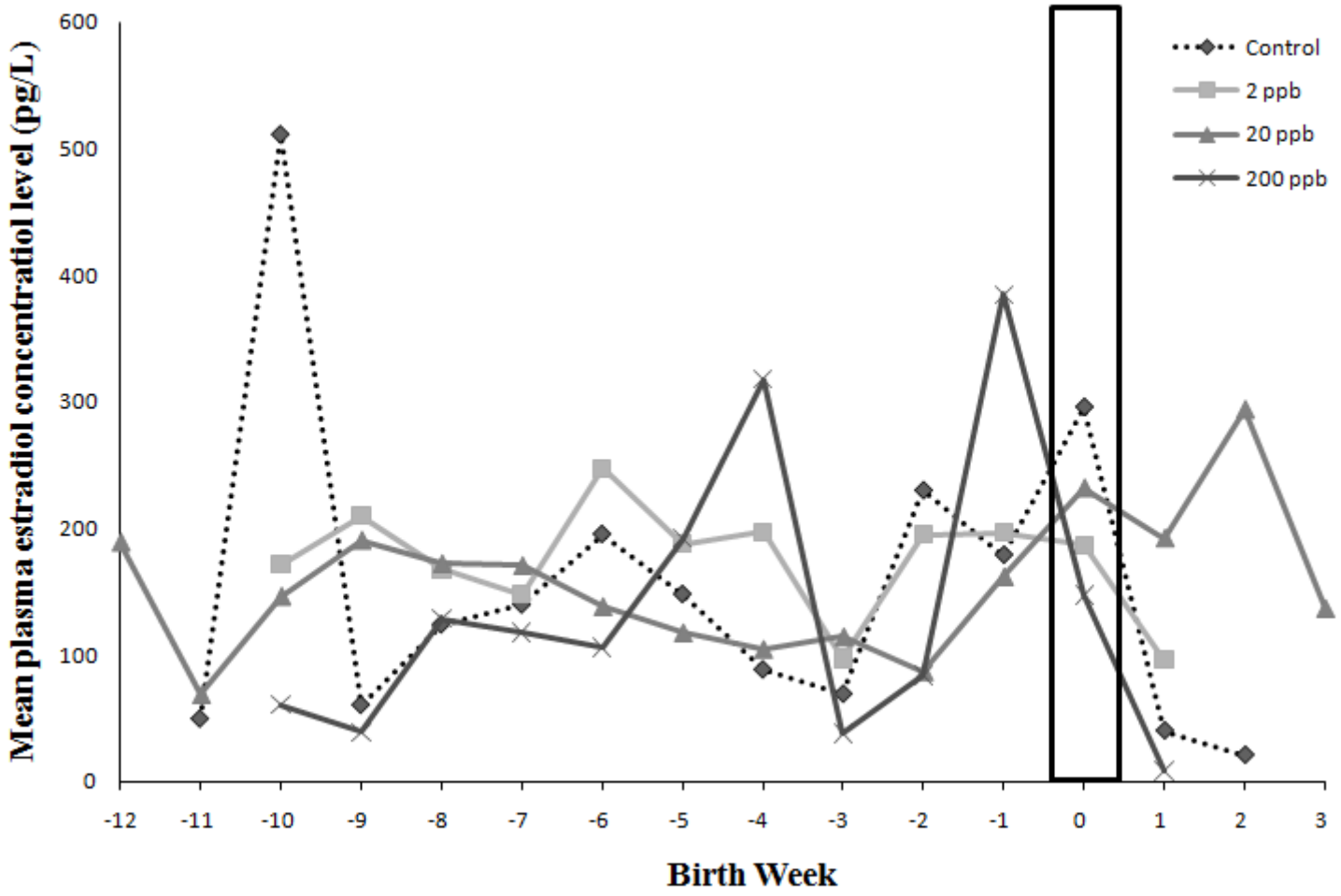

Figure 12. Mean plasma estradiol concentration in female Northern Watersnakes (*Nerodia sipedon*) exposed to one of four atrazine treatments as a function of the week of gestation. Week 0 (indicated by black box) is the week the female gave birth.

Appendix I

Solid phase extraction protocol and purpose of each step

Step	Purpose
1. *Sample Preparation* a. Thaw plasma on ice b. Pipette 70 µL plasma into 10 ml of dH_2O	Mixing the plasma sample in water allows for the sample to be effectively collected in the SPE tube.
2. *Solvation* a. Add 3 ml HPLC-grade EtOH b. Open valves and allow EtOH to flow through until 1 mm remains	EtOH wets the surface and removes any contamination on the tube surface.
3. *Equilibration* a. Add 5 ml dH_2O to columns (2x) b. Open valves and allow dH_2O to flow through until 1 mm remains	The water is able to wet the silica to further prepare for the sample.
4. *Load sample* a. Add ≈ 5 ml of sample to columns (2x) b. Open valves and allow sample to flow through until 1 mm remains	The steroids form van der Waal bonds (nonpolar:nonpolar) bonds with the C_{18}-bonded silica. The water flows through due to its polarity.

5. *Interference Elution*
    a. 5 ml dH_2O to columns (2x)
    b. Open all valves and allow to flow through for 5 min using vacuum to ensure all dH_2O is removed

The impurities in the sample are washed away using water. The other compounds are washed into the collection tubes.

6. *Sample Elution*
    a. Add 5 ml 90% HPLC-grade MeOH to columns and soak for 2 min
    b. Allow sample to drip through slowly into clean tubes
    c. Turn on vacuum for 2 min to collect all MeOH

MeOH forms hydrogen bonds with silica, which breaks van der Waals interactions with the steroids, allowing them to pass into the collection tube.

7. *Sample Drying*
    a. Dry eluate under N_2 stream

This increases the drying rate. The use of N_2 ensures that the steroids will not oxidize. This method also decreases the chance of contamination.

8. Store sample at -20 °C or proceed to resuspension

9. *Resuspension*
    a. Add 100 µl MeOH directly onto bottom of vial
    b. Vortex 1 sec
    c. Add 900 µl diH_20 into vial
    d. Vortex 3 sec

This method of resuspension prepares the sample for direct application into the ELISA.

10. Shake at ≈ 2000 rpm for 1 hour

11. Store at 4 °C overnight

12. Shake at ≈ 2000 rpm for 1 hour

APPENDIX II

Effects of maternal atrazine exposure on liver lipid content of gravid female watersnakes (Colubridae: *Nerodia*) and their neonates

ABSTRACT

An emerging concern in ecotoxicology is the effect of contaminants on lipids, either through lipid metabolism or adipogenesis. There is growing evidence that the obesity epidemic in the United States may be partially due to the increase in a class of contaminants known as endocrine disruptors. Because of its role in detoxification and metabolism, the liver is a model organ in which to examine the effects of contaminants on lipids. Gravid Northern Watersnakes (*Nerodia sipedon*) were exposed to the endocrine disruptor atrazine for approximately 3 months via ingestion of atrazine-laced fish. The livers of neonate snakes that had been exposed to atrazine *in utero* were examined for total lipid content as a function of the concentration of ingested atrazine. The livers of 11 adult females were also examined. The masses of liver lipids did not differ across treatments for either the neonate or adult female subjects. The results of this study indicate that atrazine has no affect on adipogensis or the breakdown of lipids in the liver in *N. sipedon*.

INTRODUCTION

Determining the true effects of contaminants on wildlife in an ecosystem is not possible given the wide variety of environmental parameters that can affect an organism. Typical assays to determine the lethal effects of contaminants often do not give a clear picture of the long-term effects on an organism's fitness and reproductive output. For one class of contaminants, endocrine disruptors, it is particularly evident that multiple endpoints should be examined to ascertain the nature of both lethal and sublethal effects (Crews et al. 2000).

Measuring the impact of endocrine disruptors has typically employed the use of biochemical and molecular techniques (Gluth and Hanke 1985; Tabb and Blumberg 2006). Although these methods can establish links between endocrine disruptors and organismal fitness, a clearer picture of the lethal and sublethal effects of a contaminant can be ascertained, especially when combined with additional research on the same system. Lipid analysis is an emerging tool for analyzing sublethal effects of contaminants. Many contaminants are either bioaccumulated in lipids, affect lipid accumulation, or affect adipogenesis (Geyer et al. 2000; Tabb and Blumberg 2006; Wada et al. 2007). The liver is often used for these lipid analyses because it is integral in metabolism and detoxification (Köhler 1991).

Atrazine, one of the most widely applied herbicides in the United States, displays a wide variety of endocrine disrupting properties (McMullin et al. 2004; Hayes et al. 2010). Research on atrazine has revealed potency at low levels, including sublethal effects such as feminizing genetic males (Hayes et al. 2003), decreasing long-term

survival (Storrs and Kiesecker 2004; Neuman-Lee and Janzen 2010), and altering sex hormone production (Cooper et al. 2000).

An appropriate model for examining the endocrine-disrupting properties of atrazine is the Northern Watersnake (*Nerodia sipedon*), due to its high site fidelity, diet of fish and amphibians, aquatic nature, and wide geographic distribution (Hopkins 2000; Gibbons and Dorcas 2004). As part of a larger study analyzing the effects of atrazine on gravid Northern Watersnakes, the total lipid content in the livers of the female snakes following gestation was examined. The liver lipid content was also quantified from the neonates that were exposed to atrazine *in utero*. The purpose of this study was to establish another endpoint for examining the sublethal endocrine-disrupting properties of atrazine.

MATERIALS AND METHODS

Specimen collection

In May and June of 2009, 25 female and 16 male Northern watersnakes were collected from Lake Mattoon (Cumberland Co.) and Lake Charleston (Coles Co.). Besides being used as reservoirs for the towns of Mattoon and Charleston, Illinois, respectively, the lakes are utilized recreationally for fishing and boating. The watersheds for these two lakes are bordered primarily by agricultural fields. The snakes were collected by hand or by noose and transported back to the lab. The individuals were weighed (±1 g), measured (snout-vent length [SVL; ±1 mm]), and marked using a medical cauterizer (Winne et al. 2006). The snakes were each kept in 30x30x60 cm

plastic cages with newspaper substrate, a rock to use for shedding, and water *ad libitum*. The cages were maintained on a thermal gradient (23 – 38 °C) and were exposed to a photoperiod of 12:12 h light:dark. Males were paired with females for three weeks to ensure insemination and males were subsequently released at their sites of capture. Females were then housed individually.

Atrazine Ingestion

The females were weighed weekly and fed 10% of their body mass, adjusted for each week. This amount was divided into two feedings per week, with two days elapsed between feedings. The females were fed minnows (*Notropis* sp.) purchased live from Pana Bait Co. (Pana, IL). Fish were injected with either distilled water (control) or atrazine solutions such that the snake ingested the appropriate concentration (2, 20, or 200 ppb). If snakes did not consume the fish within one hour of being offered, each snake was force-fed its respective meal to ensure that all subjects received the proper dose of atrazine.

Tissue Collection and Analysis

Neonates for this study were gathered in one of two ways. Most of them (59%) were born to female snakes receiving the treatment. Several females died near the end of their gestation such that other fully-developed neonates (41%) were removed from the mother's oviduct post-mortem. Clutches that did not have at least one fully-developed neonate (as determined by the presence of complete scalation) were not analyzed for liver lipid content. Additionally, neonates that survived longer than 2 days post-partum were also not included in these analyses because these neonates were fed, and the lipid content in their liver is not comparable to the other neonates. Upon birth (or removal from the

mother's oviduct), the mass, SVL, and sex (determined by probing) was recorded for each neonate snake. They were marked individually and frozen at -20 °C for further analysis.

To obtain liver tissue, each neonate snake was thawed and its body cavity opened with a mid-ventral incision from the cloaca to the heart. The entire liver was removed and placed in a glass vial and frozen (-20 °C) until the lipid extraction procedure was performed. After thawing each liver sample, it was transferred to a 1.5 ml centrifuge tube and the wet mass was recorded (± 0.001 g; values reported below are means of two weighings). The tubes were placed in an oven at 60 °C for 72 h. The liver was then re-weighed to obtain the dry mass (± 0.001 g; values reported below are means of two weighings).

After drying, 500 µl of a 2:1 chloroform:methanol solution was added to the livers. The liver samples were homogenized by manually grinding the tissue with a plastic grinder for 5 min. The samples were then centrifuged at 14,000 rpm for 5 min. The supernatant was removed and placed in a clean vial, to which 300 µl of the chloroform:methanol solution was added to the pellet. The pellet was rehomogenized and centrifuged again at the same speed and duration. The supernatant was removed and added to same vial containing the original supernatant. The supernatant was dried for 24 h at ambient temperature, and then dried for 24 h at 60 °C. The vials were weighed to determine the lipid mass (± 0.001 g; reported as a mean of two weighings).

Using a similar dissection, the livers were acquired from female snakes that died during, or immediately following, gestation; these samples were frozen (-20 °C). The tissue was prepared in a similar fashion to that described above, with the exception of

having 5 ml of the 2:1 chloroform:methanol solution added before the initial homogenizing. The second homogenization was performed with 3 ml of the solution. The female livers were dried for 24 h at ambient temperature and then dried for 48 h at 60°C.

Statistical Analyses

Values of neonate and adult female liver lipid masses were normally distributed. An analysis of co-variance (ANCOVA; Proc-GLM in SAS, SAS Institute, Cary, NC) was used to detect differences in the lipid mass from neonate livers as a function of atrazine treatment, and as a function of birth-female, with female mass at time of capture as a covariate. A similar ANCOVA was used to compare lipid masses of livers from the adult females as a function of treatment group, with the female mass at time of capture as a covariate. The significance level of all statistical comparisons was set at $\alpha = 0.05$.

RESULTS

There was no effect of atrazine treatment on the lipid mass of neonate livers ($F_{3,12} = 0.52$, $p = 0.68$; Table 1). There was also not an effect of treatment on the lipid mass of female livers ($F_{3,5} = 3.05$, $p = 0.13$; Table 2).

DISCUSSION

Using liver lipid mass as a metric for determining the sub-lethal effect of a contaminant on an organism can provide a broader perspective of the true environmental impact of the contaminant. The results of this study do not reveal any effect of atrazine on the lipids of livers in either adult female or neonate watersnakes. Because atrazine exposure influenced other aspects of watersnake physiology and fitness (see body of thesis, above), it is possible that the effects of this contaminant do not manifest themselves in liver tissue during either gestation or embryonic development.

The females used for the liver analysis were individuals that had died during or immediately following gestation (within 1 week). Necropsies revealed several possible causes of death. The differing causes of death could have impacted the lipid content of the liver, especially because infection can impact lipid metabolism (Khovidhunkit et al. 2004). A second problem with analyzing the lipid content in captive snakes is the propensity for individuals to develop steatosis, a type of fatty necrosis. Captive snakes are especially prone to this condition due to a lack of exercise and a steady diet (Schaffner 1998).

There has been some research on the effects of contamination on the histopathology of livers in snakes. Ganser et al. (2003) analyzed the effect of the ingestion of coal-ash contaminated prey on the livers of the Southern Watersnake (*Nerodia fasciata*). When compared to those from non-contaminated sites, snakes from contaminated areas had a higher incidence of collagen fibers that led to liver fibrosis in many of the subjects. Additionally, the individuals from a non-contaminated area had larger lipid droplets and smaller clusters of lipoid material. In place of quantifying liver

lipid mass in snakes exposed to atrazine (as was done in this study), the use of histological techniques, or greater sample sizes within treatments, could have revealed a pattern similar to that observed by Ganser et al. (2003).

Various physiological studies have revealed that organisms developing while exposed to endocrine disruptors have higher lipid content (Verma and Tonk 1983; Grun et al. 2006), although the mechanism is uncertain. The possible explanations for a perturbation in the lipid content of the liver include an increase in adipogenesis, or that the affected individuals are less able to break down lipids (Köhler 1991; Wada et al. 2007). Although increased lipid content might not necessarily have negative impacts for neonate snakes (*e.g.*, fat deposition could fuel future growth), the improper control of adipogenesis and/or lipid breakdown can be detrimental. Additionally, a disruption in an organism's physiology is likely linked to a suite of other negative impacts, such as an inability to metabolize steroid hormones (Tabb and Blumberg 2006).

Because atrazine is known to manifest a variety of effects of different physiological systems, and watersnake exposure to this contaminant via prey ingestion is realistic in a natural ecosystem, further research should examine other possible endpoints to evaluate the range of impacts that atrazine has on semi-aquatic reptiles. The implications of these findings should be considered not only with regard to regulating the appropriate use of atrazine, but also when analyzing the effects of other aquatic contaminants.

Acknowledgments

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REFERENCES

Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK. 2000. Atrazine disrupts the hypothalamic control of pituitary-ovarian function. Toxicological Sciences 53(2):297-307.

Crews D, Willingham E, Skipper J. 2000. Endocrine disruptors: Present issues, future directions. Quarterly Review of Biology 75(3):243-260.

Ganser L, Hopkins W, Neil L, Hasse S, Roe J, Sever D. 2003. Liver histopathology of the Southern Watersnake, *Nerodia fasciata fasciata*, following chronic exposure to trace element-contaminated prey from a coal ash disposal site. Journal of Herpetology 37(1):219-226.

Geyer H, Rimkus G, Scheunert I, Kaune A, Schramm K-W, Kettrup A, Zeeman M, Muir D, Hansen L, Mackay D. 2000. Bioaccumulation and occurrence of endocrine-disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms including humans. In: Bioaccumulation vol 2J New Aspects and Developments. Beek B, editor. Berlin: Springer. pp 1-166.

Gibbons JW, Dorcas ME. 2004. North American Watersnakes: A Natural History. Norman, Okla: University of Oklahoma Press.

Gluth G, Hanke W. 1985. Comparison of physiological changes in carp, *Cyprinus carpio*, induced by several pollutants at sublethal concentrations. I. The dependency on exposure time. Ecotoxicology and Environmental Safety 9:179-188.

Grun F, Watanabe H, Zamanian Z, Maeda L, Arima K, Cubacha R, Gardiner DM, Kanno J, Iguchi T, Blumberg B. 2006. Endocrine-disrupting organotin compounds are

potent inducers of adipogenesis in vertebrates. Molecular Endocrinology 20(9):2141-2155.

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environmental Health Perspectives 111(4):568-575.

Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, Adame L, Chan E, Buchholz D, Stueve T and others. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proceedings of the National Academy of Science: USA 107(10):4612-4617.

Hopkins W. 2000. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. Environmental Toxicology and Chemistry 19(10):2391-2393.

Khovidhunkit W, Kim M-S, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. 2004. Effects of infection and inflammation on lipid and lipoprotein: Metabolism mechanisms and consequences to the host. Journal of Lipid Research 45(7):1169-1196.

Köhler A. 1991. Lysosomal perturbations in fish liver as indicators for toxic effects of environmental pollution. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 100(1-2):123-127.

McMullin T, Andersen M, Nagahara A, Lund T, Pak T, Handa R, Hanneman W. 2004. Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-

Dawley rats without changing estrogen receptor action. Toxicological Sciences 79:278-286.

Neuman-Lee L, Janzen FJ. 2010. Environmentally relevant levels of atrazine exposure at the onset of embryonic development impacts post-hatching behavior and survivorship of neonatal turtles. Herpetologica 66:in press.

Schaffner F. 1998. The hepatic system: The liver. In: Biology of the Reptilia, vol. 19 (Visceral organs). Gans C, Gaunt AS, editors. Ithaca, NY: Society for the Study of Amphibians and Reptiles. pp 485-531.

Storrs S, Kiesecker J. 2004. Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environmental Health Perspectives 112(10):1054-1057.

Tabb MM, Blumberg B. 2006. New modes of action for endocrine-disrupting chemicals. Molecular Endocrinology 20(3):475-482.

Verma S, Tonk I. 1983. Effect of sublethal concentrations of mercury on the composition of liver, muscles and ovary of *Notopterus notopterus*. Water, Air, & Soil Pollution 20(3):287-292.

Wada K, Sakamoto H, Nishikawa K, Sakuma S, Nakajima A, Fujimoto Y, Kamisaki Y. 2007. Life style-related diseases of the digestive system: Endocrine disruptors stimulate lipid accumulation in target cells related to metabolic syndrome. Journal of Pharmacological Sciences 105(2):133-137.

Winne CT, Willson JD, Andrews KM, Reed RN. 2006. Efficacy of marking snakes with disposable medical cautery units. Herpetological Review 37(1):52-54.

Table 1. Neonate Northern Watersnake (*Nerodia sipedon*) pooled means (± 1 SE) of liver lipid masses (g) after exposure to atrazine *in utero*.

Treatment level	n	Mean
Control	5	0.006 ± 0.002
2 ppb	4	0.008 ± 0.003
20 ppb	5	0.005 ± 0.001
200 ppb	2	0.005 ± 0.0002

Table 2. Means (± 1 SE) of liver lipid masses for Northern Watersnakes (*Nerodia sipedon*) after ingesting atrazine during gestation period.

Treatment level	n	Mean
Control	3	0.023 ± 0.004
2 ppb	2	0.023 ± 0.009
20 ppb	2	0.042 ± 0.042
200 ppb	4	0.006 ± 0.010